



***In vitro* invasive ability of X and V factor dependent
Haemophilus species across different cell types**

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Masters by Research Thesis

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School of Health Sciences

Declaration of Originality

This thesis entitled “*In vitro* invasive ability of X and V factors dependent *Haemophilus* species across different cell types”, describes original research conducted by the candidate within the School of Health Sciences at the University of Tasmania, and contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material has previously been published or written by another person except where due acknowledgment is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

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Statement of Co-Authorship of Jointly Published Work

Manuscript 1:

I was the primary author and lead investigator of manuscript 1, which comprises Chapter 3 of this thesis and was published as:

Neeraj Kumar Singh, Dale A. Kunde, Stephen G. Tristram (2016). Effect of epithelial cell type on *in vitro* invasion of nontypeable *Haemophilus influenzae*. *J. Microbiol. Methods*; 129: 66-69.

Manuscript 2:

I was the primary author and lead investigator of manuscript 2, which comprises Chapter 4 of this thesis and was published as:

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Statement of Candidature Contribution to Thesis

This thesis comprise of research investigations where the candidate **Neeraj Kumar Singh** is the lead investigator, however the following people also contributed to the published and non-published work contained within this thesis as follows:

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Dr. Dale Kunde (School of Health Sciences, University of Tasmania): Assisted with research project design, experimental techniques, data analysis and manuscript revisions.

Dr. Stephen Tristram (School of Health Sciences, University of Tasmania): Assisted with techniques performed in microbiology laboratory and manuscript revisions.

Specific contributions to Published Chapter 3:

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Stephen G. Tristram	10%	5%	10%

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Yours Sincerely,

Neeraj Kumar Singh

General Abstract

Nontypeable *Haemophilus influenzae* (NTHi) is an opportunistic human pathogen responsible for diseases such as exacerbations of chronic bronchitis, community acquired pneumonia, otitis media and occasionally conjunctivitis. *H. haemolyticus* is closely related to NTHi and shares the same respiratory niche as a commensal, but is not an opportunistic respiratory pathogen. Both NTHi and *H. haemolyticus* can acquire resistance to β -lactam antibiotics via mutations to the *ftsI* gene and associated amino acid substitutions in penicillin binding protein 3 (PBP3) and the prevalence of such resistant strains is increasing worldwide. The factors associated with pathogenicity of NTHi are complicated, but can include mucociliary interactions, attachment to respiratory mucosa, evasion of mucosal immunity, and invasion of respiratory epithelial cells. There is significant *in vivo* and *in vitro* evidence that NTHi can invade and persist within host epithelial cells leading to the hypothesis that this allows the organism to avoid the normal immune response and establish a persistent reservoir for infection. Despite increased understanding of some mechanisms involved with invasion, the relationship between intracellular NTHi and pathogenesis is still unclear and many studies have shown enormous strain-to-strain variation in the *in vitro* invasive ability of clinical isolates. One of the limitations in understanding the relationship between intracellular NTHi and pathogenesis is the lack of a standardised model for studying invasion, as a very large range of both respiratory and non-respiratory, and primary and immortal cell lines have been used, often without explanation or justification. It is unclear whether an isolate that shows *in vitro* invasion in one cell type will be similarly invasive in another cell type, and this makes comparisons between studies very difficult.

The aims of this thesis were to investigate the effect of respiratory cell types and presence of altered PBP3 on invasion rate of NTHi and *Haemophilus haemolyticus*. To investigate our aims, we established a large collection of NTHi and *H. haemolyticus* isolates where the identity had been confirmed previously using a validated PCR algorithm as either NTHi (being positive

for *hpd#3* or *fucK* and negative for *sodC*) or *H. haemolyticus* (positive for *sodC* and negative for both *hpd#3* and *fucK*). In this working collection, NTHi isolates were collected from four different sites and clinical conditions such as otitis media, conjunctivitis, lower respiratory tract infection and normal oropharyngeal flora, whereas *H. haemolyticus* isolates were recovered from the oropharynx of healthy individuals.

These isolates were tested for invasion using the gentamicin survival assay with immortalised BEAS-2B (Sigma-Aldrich); isolated from normal human bronchial epithelium of non-cancerous individuals NHBE (Lonza); isolated from epithelial lining of airways above bifurcation of the lungs A549 (Public Health England); epithelial lung carcinoma cells derived from 58 year old Caucasian male and NCI-H292 (ATCC); muco-epidermoid pulmonary carcinoma cells derived from 32 year old female epithelia cell lines. Cell lines were grown and maintained in LHC8 (Gibco), BEGM (Lonza), DMEM growth medium (Sigma-Aldrich) supplemented with 2Mm Glutamine and 10% Foetal Bovine Serum (FBS), and RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FBS respectively at 37°C in 5% CO₂.

Chapter 3 is a detailed study of the invasive ability of NTHi, possessing normal or PBP3, using four different and widely used respiratory cell types: BEAS-2B, NHBE, A549, and NCI-H292. The focus of this study was to evaluate if there is any difference in invasive ability of NTHi isolates individually, collectively and between isolates with normal and altered PBP3 across each cell type. The results showed that NTHi invasion of respiratory epithelial cells *in vitro* is both strain dependant and influenced significantly by the type of cell lines used and also confirmed the previous suggestions, provided by Okabe *et al.* (2010) and Atkins *et al.* (2014), that isolates with altered PBP3 possess more invasive ability compared to isolates with normal PBP3. Furthermore, the association between altered PBP3 and increased invasion was conserved across each cell line.

H. haemolyticus is considered to be a non-pathogenic commensal of the respiratory tract but little information is available on its ability to invade epithelial cells *in vitro*. If *in vitro* invasion is an indicator of ability for *in vivo* invasion and is important in the pathogenesis of NTHi infection, then *H. haemolyticus* would be expected to be comparatively non-invasive. As a result,

Chapter 4 examined the invasive ability of *H. haemolyticus* to the BEAS-2B cell line. The invasion rate of 20 *H. haemolyticus* isolates were tested with BEAS-2B cell line and then 5/20 isolates were selected to test their invasion rate with other respiratory cell types used previously in this study. The results confirmed that non-invasiveness of *H. haemolyticus* isolates is consistent with their inability to cause respiratory infections.

In conclusion, this thesis has demonstrated the significant variability of invasion results across different epithelial cell lines, highlighting the importance of the choice of cell type in invasion assays as a confounding factor, as the ability of NTHi to invade respiratory epithelial cells *in vitro* is both strain dependant and influenced significantly by the respiratory cell types. Furthermore, we have shown that the association between altered PBP3 and increased invasion is conserved across all the respiratory epithelial cell types used in this study. Finally, this thesis also revealed the inability of *H. haemolyticus* isolates to invade respiratory epithelial cell types *in vitro*, and suggests that this is consistent with their inability to cause opportunistic respiratory tract infections.

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List of Abbreviations

AECB	Acute Exacerbations of Chronic Bronchitis
AOM	Acute Otitis Media
APC	Antigen-presenting cells
BEGM	Bronchial Epithelial Growth Medium
BLNAR	β -Lactamase-Negative Ampicillin-Resistant
BLNAS	β -Lactamase-Negative Ampicillin-Susceptible
CAP	Community Acquired Pneumonia
CFU	Colony Forming Units
ChoP	Phosphorylcholine
CLSD	Chronic Suppurative Lung Disease
COPD	Chronic Obstructive Pulmonary Disease
gBLNAR	Genomic BLNAR
Hap	<i>Haemophilus</i> adhesion and penetration protein
<i>H. haemolyticus</i>	<i>Haemophilus haemolyticus</i>
Hia	<i>Haemophilus influenzae</i> adhesion
Hib	<i>Haemophilus influenzae</i> type B
<i>H. influenzae</i>	<i>Haemophilus influenzae</i>
LOS	Lipooligosaccharide
MIC	Minimum Inhibitory Concentration
MLST	Multi Locus Sequence Typing
NTHi	Nontypeable <i>Haemophilus influenzae</i>
OaPA	Opacity-associated protein A
OMP	Outer Membrane Protein
PBB	Protracted Bacterial Bronchitis
PBP	Penicillin Binding Protein

Summary of Thesis

Background

Nontypeable *Haemophilus influenzae* (NTHi) is an opportunistic pathogen causing both acute and chronic respiratory infections in humans. *Haemophilus haemolyticus* is closely related to NTHi and shares the same respiratory niche, but is considered to be a non-pathogenic organism. Both NTHi and *H. haemolyticus* can acquire resistance to β -lactam antibiotics via mutations to the *ftsI* gene and associated amino acid substitutions in penicillin binding protein 3 (PBP3) and the prevalence of such resistant strains is increasing worldwide. A number of NTHi isolates has been shown to be able to invade respiratory epithelial cells *in vitro*, and it has been suggested that this may be a virulence attribute, particularly in persistent infections where the intracellular environment would protect the bacterial cells from both the host immune response and antibiotic exposure.

Although many studies have demonstrated the intracellular survival of NTHi, the relationship between intracellular NTHi and pathogenesis is still unclear. One of the constraints in understanding the relationship between intracellular NTHi and pathogenesis is the lack of a standardised model for studying invasion and the use of different cell types, often without explanation or justification. Furthermore, while the invasive ability of NTHi including increased invasion of NTHi strains with altered PBP3 has been studied, little information is available on the invasive ability of *H. haemolyticus* strains. Therefore, this thesis aims to address the specific gaps in the knowledge surrounding our understanding of the association between invasion and pathogenicity of NTHi and *H. haemolyticus*.

Chronology of Works and Thesis Organisation

Chapter 1 is a review of the current literature summarising the background information on the main topics of the thesis. This includes a general overview of NTHi as a human pathogen and a detailed review of factors associated with the adhesion and invasion of NTHi. Finally, this chapter finishes with the major aims of the thesis.

Chapter 2 presents a detailed description of methods and materials that have been used to investigate the specific aims of this thesis. This chapter includes the culture and maintenance conditions of bacterial isolates and human respiratory epithelial cells, invasion assay procedure and the use of statistical methods for data analysis.

Chapter 3 is an edited version of the first manuscript which aimed to investigate the invasive ability of NTHi across a range of respiratory epithelial cells. The hypothesis in this study was that the invasion rate of NTHi would vary from one cell type to another, as each cell type possesses specific characteristics.

This manuscript has been published as:

Neeraj Kumar Singh, Dale A. Kunde, Stephen G. Tristram (2016). Effect of epithelial cell type on *in vitro* invasion of nontypeable *Haemophilus influenzae*. *J. Microbiol. Methods*; 129: 66-69.

The novel findings from this study suggest that the choice of cell type in invasion assays is a confounding factor in *in vitro* invasion, as the ability of NTHi to invade respiratory epithelial cells *in vitro* is both strain dependant and influenced significantly by the respiratory cell types used. Furthermore, the association between altered PBP3 and increased invasion has also been strengthened by this study and extends to a range of respiratory epithelial cell types used in this study.

Chapter 4 presents an edited version of the second manuscript, which aimed to investigate the invasive ability to *H. haemolyticus* across a range of respiratory cell types. The hypothesis of this study was that if *in vitro* invasion is an indicator of ability for *in vivo* invasion and is important in the pathogenesis of NTHi infection, then isolates of *H. haemolyticus* will be comparatively non-invasive.

An unedited version of this manuscript is currently in press as:

Neeraj Kumar Singh, Dale A. Kunde, Stephen G. Tristram (2016). Inability of *H. haemolyticus* to invade respiratory epithelial cells *in vitro*. *J. Med. Microbiology*. In press. doi: 10.1099/jmm.0.000349.

The novel findings from this study suggest that the inability of *H. haemolyticus* isolates to invade respiratory epithelial cells is consistent with their inability to cause opportunistic

infections. This finding further increased the understanding of association between invasion and pathogenicity in NTHi.

Chapter 5 holds an overall discussion and the concluding remarks regarding the novel findings of this thesis. This chapter also addresses the potential limitations of the research studies undertaken and the future work that can further enhance the understanding of association between invasion and pathogenicity in NTHi.

Finally, as this thesis includes two manuscripts that have been reformatted to meet the criteria for a thesis submission at the University of Tasmania, there are some important points to note;

1. All the referencing styles have been changed to the author-date style of Harvard Referencing and a complete reference list is presented at the end of the thesis as chapter 6.
2. Supplementary data of the published manuscript has been notified as “*submitted as supplementary data in the associated publication*” in the chapter manuscript of the thesis.
3. Some abbreviations and terms are repeated between chapters, such that chapters may be read as independent manuscripts.

Chapter 1.0 – General Introduction and Literature Review

The genus *Haemophilus* represents a group of Gram-negative *coccobacilli* which colonise humans and animals exclusively. This genus gets its name due to its characteristic requirements on blood derivatives for growth. Although there are a number of *Haemophilus* species that colonise humans, including *Haemophilus parainfluenzae*, *Haemophilus ducreyi*, and *Haemophilus aphrophilus*; *Haemophilus influenzae* is the leading human pathogen amongst them (Baron S, 1996).

1.1 *Haemophilus influenzae*

H. influenzae was first recognized by Pfeiffer in 1892, who misunderstood it as a cause of influenza. It is a pleomorphic microbe that colonises the upper respiratory tract of the host and exclusively causes systemic and respiratory infections in humans. *H. influenzae* requires X (hemin) and V (nicotinamide adenine dinucleotide (NAD)) factors for aerobic growth, although it can also grow under anaerobic conditions and can be cultured in the laboratory on chocolate agar for research purposes (King, 2012). *H. influenzae* has been classified into two groups based on the presence or absence of a polysaccharide capsule; strains with a capsule are known as “typeable or encapsulated” strains, and strains without a capsule are known as “nontypeable or non-encapsulated” strains.

1.1.1 Encapsulated *H. influenzae*

Encapsulated strains can be divided into six groups designated a-f on the basis of serological reactions with antigens on the capsule. Encapsulated strain type b (Hib) is the most clinically important and is responsible for systemic infections such as pneumonia, meningitis, and bacteraemia, especially in children. Hib was a serious concern prior to the introduction of conjugate vaccines and was responsible for three million illnesses and over 386,000 deaths

per year, but the number of cases has been reduced greatly since the introduction of vaccines in countries that have implemented a Hib vaccination program (Tristram et al., 2007).

1.1.2 Nontypeable *H. influenzae*

Nontypeable *Haemophilus influenzae* (NTHi) is a non-capsulated opportunistic pathogen that is associated with respiratory infections and predominantly infects immunocompromised patients. NTHi has an extremely heterogeneous population structure and highly variable surface antigens that can be confirmed by electrophoresis or enzymatic analysis of the outer membrane. NTHi can be classed into biotypes using ornithine decarboxylase, urease, and indole (King, 2012). Strain typing can also be done by multi-locus sequence typing (MLST) pattern analysis where classifications are made based on the genetic variability of seven housekeeping genes (Meats et al., 2003).

1.1.2.1 Nontypeable *Haemophilus influenzae* (NTHi) diseases

NTHi is a commensal of the normal upper respiratory tract flora. Colonisation begins in infancy, with up to 20% of children colonised in the first year of life, greater than 50% of children colonised by the age of 5-6 years, and around 75% colonised by adulthood (Howard et al., 1988; King, 2012). However, colonisation rates are higher in at-risk populations such as Aboriginal Australian infants (Watson et al., 2006). During this dynamic process, different strains can be acquired throughout the life span of an individual. Interestingly, an adult usually carries a single strain at one time whereas children can be colonised concurrently with multiple strains (King, 2012; Murphy et al., 1999). The nasopharynx is known to be a potential reservoir of NTHi infection, from where it disseminates to the lower respiratory tract, ears and eyes during opportunistic infections (King, 2012). NTHi is predominantly associated with diseases such as community acquired pneumonia (CAP) in adults, acute otitis media (AOM), and acute sinusitis, and is frequently involved in acute exacerbations of chronic obstructive pulmonary disease (COPD) (Tristram et al., 2007).

1.1.2.1.1 Acute Otitis Media (AOM)

AOM is one of the most common infectious diseases in infants with over 700 million cases each year globally (Monasta et al., 2012). Its impact on children is enormously high in both developed and developing countries. In developed countries, for which data on the burden of this condition is more readily available, up to 80% of children in the first three years of their life will have experienced at least one episode of AOM and up to 40% will have repeated episodes of AOM by the age of seven years (Monasta et al., 2012; Teele et al., 1989; Vergison et al., 2010). *Streptococcus pneumoniae*, NTHi and *Moraxella catarrhalis* are the most common causes of bacterial AOM, being responsible for up to 40%, 20-30% and 10-20% of bacterial cases worldwide, respectively (Straetemans et al., 2004; Murphy et al., 2009). The infection starts with colonisation of bacteria in the nasopharynx and then involves subsequent dissemination into the middle ear through the eustachian tube; however, this process is usually facilitated by a preceding or concurrent viral infection (Murphy et al., 2009). Some cases of AOM will resolve spontaneously but antibiotic treatment is prescribed in many of the cases. Some of these cases will reoccur after initial treatment which may require patients to undergo repetitive treatment (Hotomi et al., 2005; Tristram et al., 2007).

1.1.2.1.2 Conjunctivitis

Bacterial conjunctivitis is frequently associated with NTHi. Children younger than 6 years of age are prone to NTHi-triggered acute conjunctivitis (Block et al., 2000; Høvdig, 2008). It has been observed that *H. influenzae* conjunctivitis leads to the development of concurrent infections and generally co-exists with reoccurring otitis media (Block et al., 2000). Previous studies have shown that the *H. influenzae* strains associated with conjunctivitis were similar to strains isolated from the nasopharynx in the same patients, suggesting that the nasopharynx is a reservoir for these strains (Trottier et al., 1991).

1.1.2.1.3 Protracted Bacterial Bronchitis/Chronic Suppurative Lung Disease/Non-cystic Fibrosis Bronchiectasis

Protracted bacterial bronchitis (PBB), chronic suppurative lung disease (CLSD) and bronchiectasis are frequent lung diseases in children worldwide (O'Grady et al., 2013). It is difficult to distinguish these diseases individually due to overlap in signs and symptoms, including chronic wet cough, and thus these conditions are collectively termed CLSD in clinical settings (Chang et al., 2010; Goyal et al., 2016; Zgherea et al., 2012). NTHi is the leading pathogen recovered from the lower respiratory tract of children with CLSD and accounts up to 49% of cases, compared to other pathogens such as *S. pneumoniae* (20%) and *M. catarrhalis* (17%) (O'Grady et al., 2013; Zgherea et al., 2012).

1.1.2.1.4 Community Acquired Pneumonia (CAP)

Pneumonia is a disease of the lung parenchyma with symptoms including chest pain, fever, productive cough, tachypnoea and dyspnoea. In CAP, the pathogens usually originate from the patient's own upper respiratory tract commensal flora (Driver, 2014; Thiem et al., 2011). CAP is one of the important causes of morbidity and mortality throughout the world, especially in elderly patients. NTHi is the second most common bacterial cause of CAP behind *Streptococcus pneumoniae*, accounting for 2-12% of cases of CAP worldwide (Thiem et al., 2011). NTHi triggered CAP generally occurs with other underlying lung disease such as COPD (King, 2012).

1.1.2.1.5 Chronic Obstructive Pulmonary Disease (COPD)

COPD is a chronic lung disease responsible for a high degree of mortality and morbidity worldwide, however the incidence of COPD varies from one country to another. In Australia, it is estimated that approximately 2.1 million people are living with some form of COPD and by 2050, this figure is expected to rise to 4.5 million (The Australian Lung Foundation, 2012). Exacerbations of COPD can be life-threatening and frequently appear as increased cough,

airway inflammation, and/or dyspnoea, and increased inflammatory markers in the respiratory tract (MacIntyre and Huang, 2008; Sethi et al., 2002; Siddiqi and Sethi, 2008). NTHi infection is among the most frequent causes of acute exacerbations of COPD and accounts for 25 to 80% of cases worldwide (King, 2012). Patients with COPD are usually colonised with more than one NTHi strain, but it is the acquisition of new strains that appears to trigger the potentially fatal acute exacerbations of COPD (Murphy et al., 1999; Sethi et al., 2002). This suggests that the heterogeneity of NTHi strains significantly compromises the efficacy of the immunity shield, as pre-existing surface specific antibodies, developed over time due to infections from other strains, may not be able to recognise the surface antigen of newly acquired strains (Siddiqi and Sethi, 2008; Yi et al., 1997).

1.1.2.2 β -lactamase Negative Ampicillin Resistance

Antibiotics are the common line of treatment for NTHi associated infections; however, the resistance to β -lactams like ampicillin, a commonly used antibiotic in NTHi infections, has increased greatly over time (Rao et al., 1999). β -lactams interrupt the actions of penicillin-binding proteins (PBP) responsible for the formation of the cell wall in bacterial cells, and this disruption in cell wall synthesis leads to the destruction or growth inhibition of the organism (Williamson et al., 1986).

H. influenzae strains can acquire resistance to β -lactams via β -lactamase production or due to alterations in penicillin binding protein 3 (PBP3) binding sites. Alterations in PBP3 binding sites result in reduced susceptibility and binding affinity to β -lactam antibiotics. Strains with such characteristics and lacking a β -lactamase are known as β -lactamase-negative ampicillin-resistant (BLNAR) (Tristram et al., 2007).

The prevalence of BLNAR strains is variable according to country and also to the criteria used to designate a strain as BLNAR, but is notably high in countries such as Japan (60%) and Spain (40%) (Kakuta et al., 2016; King, 2012; Puig et al., 2013). These strains possess a range of ampicillin minimum inhibitory concentration (MIC) values and as a result, it is difficult

to assess the clinical significance of the resistance or reduced susceptibility and the relevance to treatment efficacy. Clinically, treatment failure may occur due to resistance itself, or it may be partly attributable to the ability of NTHi to invade and survive within the host cells, a place where bacteria can be shielded from the immune system and extracellular antibiotic treatment (Garcia-Vidal et al., 2009; Murphy et al., 2004). In these cases, patients should be treated with antibiotics that can penetrate human cells such as macrolides, tetracyclines and quinolones to prevent intracellular survival and persistence in the host (King, 2012).

1.1.2.3 NTHi Recurrence and Persistence

NTHi has previously been considered an extracellular pathogen, but the frequency of recurrence and persistence of disease raises the concern that bacteria might be surviving intracellularly. The ability of NTHi to survive intracellularly has subsequently been confirmed by many studies, both *in vitro* and *in vivo* (Clementi and Murphy, 2011). One study reported that NTHi usually colonised the lower respiratory tract of COPD patients, and that after repetitive episodes of negative culture events, the original strain was isolated suggesting that it can persist within the host (Murphy et al., 2004). The probability of re-acquiring the same strain is unlikely due to very high heterogeneity between NTHi strains. In addition, undetected presence of the strain in the mucosal layer was ruled out as the strain was easily cultured from sputum samples (Clementi and Murphy, 2011; Murphy et al., 2004).

Two possible mechanisms for NTHi persistence are sequestering within a biofilm or within host cells. The ability of *H. influenzae* to exist within a biofilm or invade into host cells has been demonstrated by many studies (Murphy and Kirkham, 2002; Tikhomirova and Kidd, 2013; Wu et al., 2014). The persistence in either form would be able to provide a strong resistance to antibiotics and the immune system compared to usual planktonic growth (Swords, 2012). This inability of antibiotics and immune molecules to penetrate these defensive mechanisms would allow the microbes to reside and survive in an undetectable phase within the host for a longer span of time. The host can subsequently act as a reservoir

for future infection. However, the emphasis of this literature review will be to explore NTHi competence in attachment, invasion and persistence within the host cells.

1.1.2.4 Colonisation and Host Immune Evasion

Colonisation enables microbes to cause infection and persist within the host. NTHi usually colonises the respiratory tract of humans as a first step to causing infections. The human respiratory tract is equipped with a variety of structural defence mechanisms such as the respiratory epithelium, and the production of mucous and the mucociliary apparatus to control bacterial colonisation by trapping and clearing the pathogens through ciliary action and sputum production (Finney et al., 2014). There are a number of factors such as smoking and other pathogenic infections that disrupt the integrity and efficiency of these structural shields and allow the NTHi to start the initial colonisation in the respiratory tract by penetrating the mucus layer (King, 2012). Outer membrane proteins (OMP) associated with adhesins like P2 and P5 can bind to mucin in the nasopharynx and have the ability to enhance the initial colonisation (Foxwell et al., 1998).

The immune system is comprised of structural mechanisms, antimicrobial peptides and innate immune cells. To avoid such mechanisms, NTHi utilises a number of strategies including biofilm formation and invasion into host cells. In an instance of NTHi infection, host antigen-presenting cells (APC) exert a strong antibody response. Upon encountering this host response, NTHi strains secrete IgA proteases, which are adept at providing resistance against IgA-mediated killing (Erwin and Smith, 2007). Once NTHi gets into the lower respiratory tract, it inhibits the mucociliary action which results in inefficient mucus clearance from airways, thus creating a suitable environment for colonisation (Janson et al., 1999; Johnson and Inzana, 1986). NTHi also possesses other important characteristics including phase variation and antigenic drift (the loss or gain of antigenic structures that prevent the immune system from recognising the microbe) to avoid mounting antimicrobial responses (Gilsdorf et al., 2004; Rao et al., 1999).

1.1.2.5 Attachment

A pivotal element in NTHi pathogenesis is the ability to bind with respiratory mucosa and epithelial cells. A number of recent studies have demonstrated NTHi attachment with a variety of cell types, but it appears that non-ciliated and damaged cells within the respiratory epithelium are the preferred place for attachment (Ketterer et al., 1999; St Geme, 2002). NTHi utilises a variety of components to establish an attachment with human respiratory cells including different proteaceous adhesins and pili.

Pili are small hair-like projections found on the surface of the bacterial cell wall; however, these structures are less common in NTHi compared to other species and are only possessed by up to 33% of strains (Sethi and Murphy, 2001). They facilitate red blood cell (RBC) agglutination and stimulate attachment to the respiratory mucosa. It has been observed that sialylated lactosylceramide derivatives and fibronectin of pili promote adherence to the respiratory cells and binding to mucin respectively (Foxwell et al., 1998; Rao et al., 1999; St Geme, 2002).

NTHi strains lacking pili can still adhere to respiratory epithelial cells and it appears that autotransporter proteins play a key role to enhance the adherence in such strains (Rao et al., 1999; St Geme, 2002). High molecular weight proteins HMW1 and HMW2 are present in up to 70-80% of NTHi strains and are the surface adhesins responsible for attachment via specialised glycoprotein receptors of respiratory epithelial cells (Foxwell et al., 1998; Rao et al., 1999). The strains lacking HMW1 and HMW2 are also found to be able to attach to respiratory epithelial cells, as adherence in such strains is controlled by an adhesion gene (*Hia*) that encodes for a high molecular weight adhesion protein (Rao et al., 1999; Sethi and Murphy, 2001). It has also been shown that NTHi strains without HMW1 and HMW2 proteins and *Hia* can still exhibit a low-level of adherence. This is mainly due to the *Haemophilus* adhesion and penetration protein (Hap), which is expressed by all NTHi strains and promotes NTHi attachment to respiratory mucosa and epithelial cells. (St Geme, 2002).

OMPs are a vital family of proteins that exist on outer membrane surface of NTHi (see table 1.1) and have been identified as important mediators of interaction with and adherence to mucin. This characteristic potentially makes them important in NTHi pathogenesis. OMPs are universal in distribution amongst all NTHi strains and exhibit significant variations in size and sequences of amino acids from one strain to another (Sethi and Murphy, 2001; St Geme, 2002). P2, a porin with high immunogenicity, is the most frequent outer membrane protein and has been shown to interact with mucin through recognition of sialic acid-containing oligosaccharides (Hansen et al., 1988; St Geme, 2002). P5 is another important OMP similar to OmpA in *Escherichia coli*. P5 is a fimbrin protein that involves formation of pilus-like appendages and stimulates adhesion by conjoining to eukaryotic cell adhesion molecules (St Geme, 2002). A surface-associated lipoprotein known as opacity-associated protein A (OapA) is also expressed by *H. influenzae* strains. This forms the transparent colony phenotype and has been shown to enhance the bacterial attachment to cultured epithelial cells (St Geme, 2002).

LOS is a key element in surface adhesion molecules that facilitates NTHi adherence with respiratory epithelial cells. The *H. influenzae* lipooligosaccharide molecular structure contains oligosaccharides rather than a repeating O antigen. It has been reported that wild-type LOS-coated polystyrene beads were able to demonstrate significantly higher adherence to human bronchial epithelial cells compared to truncated mutant LOS-coated polystyrene beads (Swords et al., 2000). Further confocal investigations showed that NTHi strains containing phosphorylcholine (ChoP) residue in LOS were co-localised with host cell platelet activating factor (PAF) receptor and the strains lacking ChoP exhibited decreased adherence and invasion. The findings of these studies suggest that ChoP promotes NTHi adherence to host cells through interaction with PAF receptor (Swords et al., 2000).

Table 1.1 List of OMPs and their roles.

OMPs	Functions	References
P1	Transport binding protein.	Bolduc et al., 2000
P2	Interaction with mucin via recognition of sialic acid-containing oligosaccharides.	St Geme, 2002
P4	Mediates bacterial adherence to pulmonary epithelial cells.	Su et al., 2016
P5	Pilus-like surface appendages formation and adherence by interacting human cell adhesion molecules.	St Geme, 2002
P6	Maintains cellular integrity of outer membrane of bacteria and mediates interaction with host cells.	Murphy et al., 2006
OMP26	Translocation of OMPs and LOS.	Riedmann et al., 2003
OapA	Transparent colony phenotype formation and bacterial attachment to cultured epithelial cells.	St Geme, 2002
Lipoprotein D (LPD)	Bacterial adherence and entry into monocytes/macrophages.	Ahren et al., 2001a
Transferrin-binding protein B	Transferrin-derived iron transport across the outer membrane.	Webb and Cripps, 1999
Protein D15	Homology to <i>E. coli CDP-diglyceride synthetase</i> .	Loosmore et al., 1997
HMW1 and HMW2	Surface adhesins that promotes adherence via specialised glycoprotein receptors of respiratory epithelial cells.	Foxwell et al., 1998
HtrA/H91A	Heat shock protein	Alonso et al., 2005

Invasion into host cells is an approach that can be used by NTHi to avoid antibiotics and the immune system of the host. Bacterial invasion into host cells depends on the interaction between bacteria and host cell receptors; however, there appear to be two primary pathways that NTHi utilises to invade human epithelial cells.

Receptor-mediated invasion appears to be the main pathway of invasion of most NTHi strains and is predominantly mediated by the PAF receptor of human respiratory epithelial cells. It has been demonstrated that wild type NTHi strains containing LOS frequently co-localise with the PAF receptor of epithelial cells (St Geme, 2002; Swords et al., 2000). As discussed above, NTHi strains containing LOS with a ChoP moiety can invade epithelial cells more aggressively than mutant strains lacking the oligosaccharide region of LOS. As LOS localises with PAF receptors of epithelial cells, it was presumed that the PAF receptor might play a significant role in NTHi invasion. This was then investigated by inhibiting the PAF receptor using a PAF receptor antagonist treatment which significantly reduced the invasive ability of wild-type NTHi isolates, but did not affect the invasive ability of LOS mutant strains even at high concentration of the PAF receptor antagonist. It was therefore suggested that some other mechanism may also contribute to NTHi invasion (Swords et al., 2001).

Macropinocytosis is a highly conserved form of endocytosis which is a receptor independent, actin driven process to stimulate membrane ruffling in eukaryotic cells and as a result internalisation of extracellular fluid materials (King, 2012; St Geme, 2002; Swords et al., 2001). It has been observed that this invasion process starts with extension of host cell microvilli and the formation of lamellipodia. These structures envelop and internalise the adherent bacteria into a membrane-bound vacuole. Confocal microscopic examination has revealed that infected bronchial epithelial cells initiate cytoskeletal rearrangement via actin polymerisation during the process of macropinocytosis and the invasion rate reduced significantly in the presence of cytochalasin D, which is an inhibitor of actin polymerisation (Holmes and Bakaletz, 1997; Ketterer et al., 1999; St Geme and Falkow, 1990). The macropinocytic process of internalisation has also been demonstrated by a marker study (Ketterer et al., 1999) and the presence of NTHi with marker into cells confirmed it as a pathway of invasion. However, NTHi isolates without markers were also observed in the bronchial epithelial cells suggesting that there is also another mechanism of host cell uptake present (Ketterer et al., 1999).

It has been shown that inhibition of macropinocytosis can hamper the invasive ability of NTHi, especially the strains with weaker expression of LOS containing ChoP moiety. Therefore, it

was concluded that macropinocytosis is the primary pathway of invasion for strains lacking ChoP, whereas receptor mediated invasion is the predominant mechanism of invasion for the majority of NTHi strains. NTHi can utilise both the pathways to invade the cells, as inhibiting either of the mechanisms was not able to completely block the NTHi entry into cells (Swords et al., 2001).

The adherence and invasion competence of NTHi into human respiratory epithelial cells has been studied by many investigators but the information about its intracellular behaviour and survival still needs to be explored. One recent study has documented that NTHi was able to persist para-cellularly in respiratory epithelial cells without damaging the cell integrity over a long period of time whereas bacteria inside vacuoles started to degrade (Ren et al., 2012). One study showed that NTHi can persist in respiratory epithelial cells in a metabolically active and non-proliferative form (Junkins et al., 2014; Morey et al., 2011). During its intracellular life cycle, NTHi travels temporarily through compartments that possess late endosome features, such as acidic pH. Most of these compartments do not fuse with lysosomes and as a result, represent a niche where organisms can persist for longer periods of time (Morey et al., 2011). Microscopic studies of the infected respiratory cells revealed the presence of NTHi clusters between epithelial cells and in the submucosa, suggesting that NTHi can pass between cells and through subepithelial space (Erwin and Smith, 2007; St Geme, 2002). This arrangement of intracellular survival is suggested as an optional habitat for persistence within the host (Clementi and Murphy, 2011).

1.1.2.7 Intracellular Survival

Studies to accurately assess the intracellular fate of NTHi are still underway. Currently, a number of culture and non-culture based assays are in use. Culture based methods, such as the gentamicin survival assay, are the most widely used to determine the *in vitro* intracellular survival of NTHi, but are not applicable when studying the viability and survival of NTHi within the host (Clementi and Murphy, 2011). Most studies have used a simple monoculture method

where the model is restricted to epithelial and bacterial cells, whereas co-culture methods that combine epithelial, bacterial and other host cells may better reflect the *in vivo* environment (Duell et al., 2011). Bacterial viability can also be determined using non-culture based methods including reverse transcription PCR, but this is not suitable to identify the intracellular location of bacteria. A range of methods are available to identify the intracellular location including Gram staining, bacterial expression of green fluorescent protein (GFP) and fluorescent *in situ* hybridisation (FISH) (Clementi and Murphy, 2011).

It is a challenging task to determine the location and viability of bacterial cells within eukaryotic cells and tissues or other complex environmental matrices; however, some sophisticated techniques have been developed to determine the identity, viability and sometimes location within the host (Amann and Fuchs, 2008; Nielsen et al., 2010). Most of these techniques measure incorporation of stable or radioisotopes or labelled substrates and are commonly used in combination with FISH, but some employ different methods such as bacterial incorporation of bromodeoxyuridine (BrdU) which is an indicator of nucleotide replication and can be identified with anti-BrdU antibody conjugates. This method has been successfully employed to identify the location and viability of intracellular *Staphylococcus epidermidis in vivo* and can be applied to study the NTHi infection models or patient samples as well (Broekhuizen et al., 2010; Clementi and Murphy, 2011).

1.1.2.8 Effect of Cell Types on Invasion

One of the limitations in understanding the relationship between intracellular NTHi and pathogenesis is a lack of a standardised model for studying invasion. Clementi & Murphy (2011) acknowledge that careful selection of both bacterial strains and host cell types for *in vitro* invasion studies is required, and yet even a brief review of the literature reveals that a very large range of both respiratory and non-respiratory, and primary and immortal cell lines has been used (table 1.2). The underlying assumption is that NTHi invades all epithelial cells in a similar fashion and that the results of the gentamicin survival assay across the cell types are comparable (Ahren et al., 2001b; Hotomi et al., 2010; Ketterer et al., 1999; López-Gómez et al., 2012; Okabe et al., 2010; Raffel et al., 2013; Swords et al., 2000). Factors such as bacterial adherence, interaction between cell receptors and adhesins, and the integrity of the cellular membrane may vary from one cell line to another due to their specific cell characteristics. Decreased bacterial adherence due to poor interaction between cell receptors and bacterial adhesins may then subsequently cause reduced bacterial entry into cells as unique cell types have their specific receptors.

Table 1.2 List of invasion studies using different cell types.

Haemophilus Isolates		Cell types		Conclusion	Authors
No. of isolates	Defining Features	Identifier	Defining Features		
5 NTHi	<i>hmw1</i> and <i>hmw2</i>	Primary Human airway epithelial cells	Nasal polyp epithelial cells	NTHi adheres to specific non-ciliated airway epithelial cells and initiate cytoskeletal rearrangement.	Ketterer et al., 1999
		SV40	Transformed bronchial epithelial cells		
		16HEB14			
8 NTHi	Wild type (4), Chop (3) and Los (1) mutants	16HBE14	Transformed bronchial epithelial cells.	A specific subset of ChoP+ LOS glycoforms could promote NTHi invasion of bronchial cells via interaction with the PAF receptors.	Swords et al., 2000
2 NTHi	Protein D ⁺ (1) and Protein D ⁻ (1)	U-937	Human monocytic leukaemia cells	Surface-expressed protein D of NTHi mediates adherence to human monocytes leading to a higher uptake of bacteria.	Ahren et al., 2001
		THP-2			
5 NTHi	Wild type	Hep-2	Human epithelial cells	NTHi ability to survive intracellularly may contribute to the persistent existence of this pathogen that result in the prolonged and intractable clinical course of AOM.	Hotomi et al., 2010
91 NTHi	gBLNAR (57) and BLNAS (34)	BEAS-2B	Immortalized bronchial epithelial cells	NTHi isolates with altered PBP3 possess higher invasive ability compared to isolates with normal PBP3.	Okabe et al., 2010
11 NTHi	Wild type	NCI-H292	Muco-epidermoid lung carcinoma cell	<i>H. influenzae</i> invasion of epithelial cells may contribute to the persistence of this microorganism in COPD and CF patients.	van Schilfgaarde et al., 1999
1 NTHi	Streptomycin resistant	NHBE	Normal human bronchial epithelial cells	NTHi Sap transporter mediates interaction with host epithelial cells	Raffel et al., 2013
		A549	Immortalized human lung carcinoma epithelial cells		
40 NTHi	BLNAS (20), gBLNAR (20)	BEAS-2B	Immortalized bronchial epithelial cells	Altered PBP3 is not directly associated with Increased invasiveness of NTHi.	Atkins et al., 2014
4 NTHi 4 H.h.	Wild type	D562	Immortalised human pharyngeal carcinoma epithelial cells	<i>H. haemolyticus</i> and NTHi exhibit distinct interactions with host cells, with different immuno-stimulatory and cytotoxic properties.	Pickering et al., 2016
		A549	Immortalized human lung carcinoma epithelial cells		

1.1.2.9 Altered PBP3 and Invasion

PBP3 is responsible for cross-linking of peptidoglycan polymers in the bacterial cell wall (Scheffers and Pinho, 2005). Similar to other PBPs, it is comprised of three functionally different domains: the short cytoplasmic domain, the membrane-spanning domain and the transpeptidase domain (Tristram et al., 2007; Zapun et al., 2008). Ampicillin resistance in the absence of β -lactamases was first discovered in *H. influenzae* in 1974 when two β -lactamase negative isolates showed resistance to ampicillin. Since then, β -lactamase negative ampicillin resistant (BLNAR) strains have frequently been observed in clinical isolates of Hib and NTHi strains. The mechanism of ampicillin resistance in β -lactamase negative isolates was first demonstrated by Parr and Bryan (1984) in *H. influenzae* Rd transformed with genomic DNA of a clinical Hib BLNAR isolate. They observed alteration in PBP3 (previously, PBP3a and PBP3b) structure that was associated with a reduction in binding affinity to ampicillin (Parr and Bryan, 1984). The mechanism was further clarified by Ubukata et al, when they showed that specific mutations in the *ftsI* gene resulted in the BLNAR defining asparagine to lysine amino acid substitution at position 526 (N526K) of PBP3 (Ubukata et al., 2001). Other *ftsI* mutations and associated amino acid substitutions have since been identified as “contributing” to resistance when present in addition to N526K (Tristram et al., 2007).

The impact of altered PBP3 on invasion was first demonstrated by Okabe *et al.* (2010) and suggested that there is an association between isolates with altered PBP3 and invasion in bronchial epithelial cells. In that study, 91 Japanese clinical NTHi isolates were screened for mutated *ftsI* genes collected from 2001 to 2005 to establish a collection of 34 BLNAS and 57 gBLNAR isolates. The invasive ability of isolates was determined by the gentamicin survival assay and the isolates classified as highly and low invasive on the basis of an arbitrary distinction of 1% invasion rate. Isolates with <1% invasiveness were designated as low invasive whereas isolates with $\geq 1\%$ invasiveness were regarded as highly invasive. Only 9 of 91 isolates were defined as highly invasive and all of them were BLNAR.

Table 1.3 Invasion rate of NTHi isolates according to amino acid substitution used by Okabe *et al.* (2010).

Group	Amino acid substitutions in PBP3					*BLNAR	No. of Isolates	Mean Invasion (%)
	M377I	S385T	L389F	R517H	N526K			
0	–	–	–	–	–	-	34	0.01
I	Ile	Thr	–	His	–	High	5	0.65
III	–	–	–	–	Lys	Low	12	0.58
IIIa	Ile	–	Phe	–	Lys	High	1	(0.05%)
IIIb	Ile	Thr	–	–	Lys	High	2	(0.38%), (1.8%)
IIIc	Ile	Thr	Phe	–	Lys	*High	33	0.95
IV	Ile	Thr	Phe	–	–	High	4	0.01

*BLNAR classification as defined by Ubukata *et al.* (2001).

*High BLNAR with maximum amino acid substitutions.

Atkins *et al.* (2014) has subsequently investigated the Okabe *et al.* (2010) findings and found that while there is an association between invasiveness and the nature of the PBP3 in the strains, altered PBP3 itself had no direct effect on invasiveness. In that study, they collected 40 isolates of NTHi and the collection was comprised of 20 isolates with normal PBP3 and 20 with altered PBP3. Isolates with altered PBP3 demonstrated a significantly higher mean invasion rate compared to isolates with normal PBP3. Furthermore, they introduced a mutated *ftsI* gene responsible for altered PBP3 into isolates with initially normal PBP3 by transformation, and then tested for invasion with BEAS-2B cells. There was no increase in invasion rate in strains with artificially introduced altered PBP3, suggesting that there may be the presence of another factor which is coregulated with naturally altered PBP3 that confers increased invasion (Atkins *et al.*, 2014).

1.1.2.10 NTHi Summary

In conclusion, it is clear from the above discussions that NTHi is one of the leading pathogens in human respiratory tract infections and can survive intracellularly within the host. NTHi invasion in human respiratory epithelial cells is a highly complex process and several studies have shown that NTHi invasion depends on various factors and mechanisms of both bacteria

and host, such as bacteria-host cell complex surface proteins, surface structures and entry pathways of host cells. This multifactorial dependence of NTHi invasiveness complicates our understanding of the pathogenesis in NTHi infections and requires further investigation.

1.2 *Haemophilus haemolyticus*

H. haemolyticus is very closely related to NTHi and shares the same upper respiratory tract niche. It is easy to distinguish NTHi and *H. haemolyticus* from other *Haemophilus* species due to requirement of both X and V blood derivatives for their *in vitro* growth, but it is a tedious task to discriminate these two on a phenotypic basis, as they possess very similar colony morphology and biochemical activity. Traditionally, the presence or absence of haemolysis on horse blood agar could be used to discriminate the two species; however, with the emergence of non-haemolytic strains of *H. haemolyticus*, the reliability of this marker is immensely hampered (Murphy et al., 2007; Sandstedt et al., 2008). Retrospective analysis by molecular detection methods have confirmed many instances where *H. haemolyticus* was erroneously identified as NTHi (Murphy et al., 2007). In one study, retrospective analysis of 490 phenotypic NTHi isolates by molecular methods demonstrated that 39.5% (102/156) of NTHi isolates from COPD patients and 27.3% (12/32) NTHi isolates recovered from the nasopharynx of children were actually non-haemolytic *H. haemolyticus*. Other studies also reported similar misidentification cases where 20.8% of presumptive NTHi recovered from throats of children and 16-21% of presumptive NTHi from lung specimens were identified as *H. haemolyticus* (Mukundan et al., 2007; Xie et al., 2006).

H. haemolyticus is generally considered to be a non-pathogen of the respiratory tract (Hariadi et al., 2015; Sandstedt et al., 2008) although there have been isolated reports of the organism being isolated from sterile site infections in immunocompromised patients (Anderson et al., 2012; Hariadi et al., 2015; Morton et al., 2012). Cellular interaction and invasion was studied and correlated with the virulence attribute of NTHi (Atkins et al., 2014; Clementi and Murphy, 2011; Okabe et al., 2010); however, there is little information available about the invasion and

intracellular activity of *H. haemolyticus*. Recently, one study has shown that *H. haemolyticus* possesses less attachment and invasive ability compared to NTHi (Pickering et al., 2016). However, this study did not mention whether they had any strains with altered PBP3 in their tested collection.

H. haemolyticus can acquire resistance to β -lactam antibiotics via mutations and associated amino acid substitutions in PBP3. The prevalence of these BLNAR strains is increasing worldwide and regular exchange of partial fragments of mutated *ftsI* gene between NTHi and *H. haemolyticus* is considered as one of the major causes of such a high increase in BLNAR strains (Takahata et al., 2007; Tristram et al., 2007; Witherden et al., 2014). The association between altered PBP3 and increased invasion is known in NTHi, but has not been investigated in *H. haemolyticus* isolates. It would be worthwhile to investigate the attachment and invasive ability of *H. haemolyticus* strains with altered PBP3 compared to strains with normal PBP3.

1.3 Chapter Summary

Traditionally, NTHi has been considered an extracellular pathogen; however, there are a number of studies confirming that it can invade and survive intracellularly in human respiratory cells. The mechanisms of cellular invasion and bacterial interaction with host cells have been defined as a highly complex processes. Some recent studies have suggested that outer membrane proteins and other surface adhesin molecules play a significant role in the invasion process through initiation of cytoskeletal changes, such as formation of microvilli and lamellipodia to facilitate NTHi internalisation into host cells.

Both NTHi and *H. haemolyticus* can acquire resistance to β -lactam antibiotics via mutations in the *ftsI* genes and the prevalence of such resistant strains is increasing worldwide. Okabe et al. (2010) provided evidence that NTHi isolates with altered PBP3 possess significantly higher adhesive and invasive ability compared to strains with normal PBP3, whereas a subsequent study by Atkins et al. (2014) suggested that increased invasiveness in naturally altered PBP3 strains is not directly associated with the altered PBP3. However, both studies

have agreed that strains with naturally altered PBP3 possess increased adhesive and invasive ability.

Despite increased understanding of some mechanisms involved with invasion, there are inconsistencies in the literature regarding our understanding of the relationship between intracellular survival of NTHi and pathogenesis, especially due to lack of standardised model and use of different cell types. Furthermore, while the invasive ability of NTHi including increased invasion of NTHi strains with altered PBP3 has been studied, there is little information available on the invasive ability of *H. haemolyticus* strains. Therefore, the exclusive aims of research studies presented in this thesis are:

- To determine the difference in invasive ability of NTHi across different cell types (Chapter 3).
- To determine the difference in invasive ability of NTHi strains with altered PBP3 compared to strains with normal PBP3 across different cell types (Chapter 3).
- To evaluate the association between non-pathogenicity and invasive ability of *H. haemolyticus* (Chapter 4).

Chapter 2.0 – Methods and Materials

2.1 Media, Culture Condition and Storage

All the bacterial isolates used in this study were grown on chocolate agar containing 5% defibrinated horse blood (Oxoid, Thebarton, Australia) and 1% Vitox (Oxoid, Thebarton, Australia), at 35°C in 5% CO₂. Heart infusion broth (Oxoid, Thebarton, Australia) containing 2% Vitox and 15mg/L NAD and haematin was used for suspended growth of NTHi isolates at 35°C in air on an orbital shaker at 150 rpm. Glycerol broth was used as storage medium to preserve all the bacterial isolates either in liquid nitrogen or frozen at -80°C. Stored isolates were recovered using standard culture conditions and subcultured at least twice for use in experiments.

2.2 Bacterial Isolates

A large collection of NTHi and *H. haemolyticus* isolates was established and the identification confirmed previously by molecular methods. A previously validated PCR algorithm was used (Witherden and Tristram, 2013), where isolates were identified as NTHi when positive for *hpd* or *fucK* and negative for *sodC* whereas *H. haemolyticus* was identified when positive for *sodC* and negative for *hpd* and *fucK*. From this collection, NTHi isolates were selected from four different sites and clinical conditions such as otitis media, conjunctivitis, lower respiratory tract infection and normal oropharyngeal flora, whereas *H. haemolyticus* isolates were recovered from the nasopharynx of healthy individuals.

NTHi isolates for subsequent invasion assays were selected from the initial collection and a working collection of 16 isolates was established (see table 2.1 for details). A working collection of 20 isolates of *H. haemolyticus* including 8 isolates with normal PBP3 and 12 isolates with altered PBP3 was also selected from initial collection and used in subsequent invasion assays. The isolates were confirmed as having normal or altered PBP3 previously, using PCR to detect the specific single nucleotide polymorphisms associated with the N526K substitution (Witherden et al., 2013).

2.1 Table List of NTHi isolates and their site of isolation.

Site of Isolation	Number of strains	Strain Identifier	Altered PBP3
Ear	4	Ci24	No
		Ci35	No
		L267	Yes
		L341	Yes
Eye	3	Ci5	No
		Ci51	Yes
		J76	No
Sputum	5	Ci8	No
		Ci34	No
		Ci49	Yes
		Ci11	Yes
		Ci17	Yes
Throat	4	NF3	No
		L60	Yes
		L236	Yes
		L227	Yes

2.3 Respiratory Epithelial Cell Types

Four different cell types were used in this study and their media, culture procedure and maintenance conditions are as below:

2.3.1 Cell Lines

BEAS-2B cell line (isolated from normal human bronchial epithelium of non-cancerous individuals) purchased from ATCC, Manassas, USA, NHBE cell line (isolated from epithelial lining of airways above bifurcation of the lungs) purchased from Lonza, Walkersville, USA, A549 cell line (epithelial lung carcinoma cells were derived from 58 year old Caucasian male) purchased from Public Health England, Salisbury, UK and NCI-H292 cell line (muco-epidermoid pulmonary carcinoma cells derived from 32 year old female) purchased from ATCC, Manassas, USA have been used to investigate the invasive ability of NTHi and *H. haemolyticus*.

2.3.2 Preparation of Collagen Coated T75 Flasks for BEAS-2B Cell Culture

BEAS-2B cell culture T75 flasks were prepared by pre-incubating with a collagen coating solution consisting of 5ml of Dilution Medium (50-9701) and 50µl of Coating Matrix (50-9701) (Gibco, Life Technologies, USA) was added to T-75 flasks. The flasks were then rocked back and forth vigorously to ensure uniform distribution of the coating matrix over the surface of the flask. The flask was incubated for 30 minutes at room temperature. The excess Coating Matrix/Dilution Medium was removed from the flask and the flask was then used for cell culture.

2.3.3 Maintenance of Cell Lines

The cells were cultured using relevant growth medium (table 2.1). Briefly, a cryovial containing 1×10^6 frozen cells was quickly thawed in a 37°C water bath and the contents of the cryovial were transferred to collagen coated T-75 flasks for BEAS-2B cells and standard T75 flasks for NHBE, A549, and NCI-H292 cells containing 10ml of pre-warmed relevant growth medium (see table 2.1). The cells were grown to confluency at 37°C in 5% CO₂. Growth medium was replaced the day after seeding and two to three times in a week thereafter. The NHBE cells were discarded after two passages, and the BEAS-2B, A549, and NCI-H292 cells were discarded after nine passages. New cells from the stock preserved in liquid nitrogen were grown and used for further assays.

A stock of the cell line was maintained for future use. Cells were stored in liquid nitrogen vapour phase in cryovials growth medium and 10% Dimethyl sulfoxide (DMSO) at a concentration of 1×10^6 cells/vial for further use.

2.3.4 Preparation of 24 Well Assay Plates

The cells were subcultured into 24-well plates for the invasion assay, the confluent cells were detached from the T-75 flasks by treatment with 5ml of TrypleEX (Gibco, Life Technologies, USA) for approximately 5 minutes at 37°C in 5% CO₂ and the cell detachment was monitored

every 1-2 minutes using an inverted microscope. TrypleEX was neutralised by addition of 10ml of pre-warmed relevant growth medium (please see table 2.1). The cells were collected in a 15ml centrifuge tube and centrifuged at 230 x g for 5 minutes to obtain the cell pellet. The TrypleEX and media were removed and the cell pellet was resuspended in 5ml of fresh pre-warmed growth media. The cells were then seeded into a 24-well plate at a recommended density/well for each cell type (see table 2.1) and incubated at 37°C in 5% CO₂ for approximately 48 hours to reach greater than 90% confluence.

Table 2.2 Cell types, media and seeding density for 24 well plates.

	Cell types			
	BEAS-2B	NHBE	A549	NCI-H292
Growth Media	LHC-8	BEGM	DMEM+2mM Glutamine +10% Foetal Bovine Serum	RPMI 1640 + 10% FBS
Media Providers	Gibco, Life Technologies, USA	Lonza, Walkersville, USA	Sigma-Aldrich, St. Luis, USA	Sigma-Aldrich, St. Luis, USA
Cell density 24 well plates	5 x 10 ⁴ cells/well	7 x 10 ⁴ cells/well	5 x 10 ⁴ cells/well	5 x 10 ⁴ cells/well

2.3.5 Respiratory Epithelial Cell Density Measurement

Automated Countess™ cell counter (Invitrogen, CA, USA) was used to count the cell density of respiratory epithelial cells. In short, the cell density count was performed by gently mixing 50µl of sample and 50µl of trypan blue (0.4%) (Sigma-Aldrich) in a 1.5ml microfuge tube, afterwards the mixture was loaded into the chamber slide. Chamber slide was then inserted into the slide inlet of Countess™ and the Count Cells button was pressed on the touch screen of the instrument to count the cells.

2.4 Invasion Assay

The invasion rate of all the bacterial isolates used in this study was measured using a gentamicin survival assay as described previously by Atkins *et al.*, (2014). Four respiratory epithelial cell types as described above were used for invasion assays. The invasion assay with each cell type was performed independently on three different days. A 24 well plate was seeded with a particular respiratory cell type at a density of 5×10^4 , 7×10^4 , 5×10^4 and 5×10^4 cells/well for BEAS-2B, NHBE, A549 and NCI-H292 cells respectively in three out of four consecutive wells (technical replicates), and incubated until greater than 90% confluence (approximately 48 hours).

A fresh culture for each bacterial isolate was prepared on chocolate agar plates and incubated for approximately 16 hours. Isolates were harvested using sterile loop and suspended in respective culture medium for each cell type at a concentration of approximately 10^6 bacteria/ml. The concentration of suspended bacterial isolates was calculated by plating serial dilutions onto chocolate agar in triplicates to determine CFU/ml (Count X).

To start the assay, wells containing the confluent monolayer of respiratory epithelial cells were washed two times with fresh pre-warmed respective cell culture medium. 1ml of previously prepared suspension of bacterial isolates was added to four wells for each isolate (three technical replicates seeded with respiratory epithelia cells, and one unseeded well) and incubated for 4 hours at 37°C in 5% CO₂. The wells were then washed three times with Hank's buffered salt solution (HBSS, LONZA, Melbourne, Australia), 1ml of fresh pre-warmed cell culture medium containing gentamicin (200mg/L) was added and the plate was re-incubated for two hours at 37°C in 5% CO₂. The wells were then washed again three times with HBSS, with the third wash plated onto chocolate agar to confirm the complete elimination of extracellular bacteria. 0.5ml of 1% Saponin was added to each well for 15 minutes in order to lyse the cell and release the intracellular bacteria. The samples were then harvested and vortexed for 1 minute before serial dilution. The serially diluted samples were plated onto pre-warmed chocolate agar for overnight incubation. The number of intracellular bacteria were

calculated by estimating the CFU/ml for each of three wells and taking the mean of three counts (count Y).

The percentage invasion rate was calculated by the formula:

$$\text{Invasion Rate} = \frac{\text{Count Y}}{\text{Count X}} \times 100$$

Each assay was performed three times for NTHi isolates and two times for *H. haemolyticus* isolates on different days and the mean invasion rate (%) was calculated from the respective biological replicates. The arbitrary distinction provided by Okabe *et al.* (2010) was used to determine the isolates with invasion rate of $\geq 1\%$ considered as highly invasive strains whereas isolates with invasion rate of $\leq 1\%$ designated as low invasive strains.

2.5 Statistical Analysis

All data analysis and figures were performed using GraphPad Prism software (version 6.0d). Analysis of all the clinical isolates was done by the mean invasion rate (%) of three biological replicates of each isolate of NTHi and two biological replicates of *H. haemolyticus* using log10 transformation. A Shapiro-Wilks test was performed to assess the normality or otherwise of the distribution of invasion rates across the isolates. Analysis of the differential invasion rate of all NTHi strains in four different cell types (BEAS-2B, NHBE, A549 and NCI-H292) was conducted using a Kruskal-Wallis test and a two-way ANOVA was performed to determine the invasion rate of each individual strain across the four cell types. In this study, different strains and four cell types were used as the independent variables whereas invasion rate (%) was used as the dependent variables. A Mann-Whitney test was performed to analyse the difference in invasion rate between NTHi strains with normal and altered PBP3 for each cell type.

P values < 0.05 were considered as statistically significant difference between the outcome of different tests.

Chapter 3.0 – Manuscript 1

Effect of epithelial cell type on *in vitro* invasion of Nontypeable

Haemophilus influenzae

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The content of this chapter is reproduced as published in the Journal of Microbiological Methods as an original research article (Submitted 15th June; revised 25th July; accepted 26th July; published 26th July 2016; 68: 66-69) with some additional information (P40, para2 and P41, para3), and the text has been reformatted to meet the thesis submission requirements.

3.1 Abstract

Nontypeable *Haemophilus influenzae* (NTHi) have been shown to have variable ability for *in vitro* invasion with a range of epithelial cells, and increased invasion of BEAS-2B cells has been associated with altered PBP3, which is concerning as these strains are increasing worldwide. The aim of the study was to investigate the effect of respiratory cell type and the presence of altered PBP3 on the *in vitro* invasion of NTHi. A collection of 16 clinical NTHi isolates was established, 7 had normal PBP3, and 9 had altered PBP3 as defined by an N526K substitution. The isolates were tested for invasion of BEAS-2B, NHBE, A549 and NCI-H292 respiratory epithelial cells *in vitro* using a gentamicin survival assay, with invasion measured as the percentage of intracellular organisms relative to the initial inoculum. The overall median invasion for the 16 NTHi isolates for cell types BEAS-2B, NHBE, A549 and NCI-H292 cells were 3.17, 2.31, 0.11 and 1.52 respectively. The differences were statistically significant for BEAS-2B compared to A549 ($P=0.015$) and A549 compared to NCI-H292 ($P=0.015$), and there were also very marked differences in invasion for some individual isolates depending on the cell type used. There was a consistent bias for invasion of isolates with normal versus abnormal PBP3: and this was statistically significant for BEAS-2B (0.07 to 9.90, $P=0.031$) and A549 cells (0.02 to 1.68, $P=0.037$). These results show that NTHi invasion of respiratory epithelial cells *in vitro* is both strain dependant and influenced significantly by the cell line used, and that the association between altered PBP3 and increased invasion is conserved across multiple cell lines.

3.2 Introduction

NTHi is a commensal of the human nasopharynx and is also associated with a large burden of disease attributable to its role in a range of opportunistic infections, such as sinusitis (41% of cases in children), otitis media (55-95% of cases in children), community acquired pneumonia (20-94% of cases) and acute exacerbations of chronic obstructive pulmonary disease (>90% of cases) (Clementi and Murphy, 2011; King, 2012; López-Gómez et al., 2012;

Van Eldere et al., 2014). Although NTHi has traditionally been considered an extracellular pathogen, there is significant *in vivo* and *in vitro* evidence that the organism can invade and persist within host epithelial cells (Clementi and Murphy, 2011; Ketterer et al., 1999; King, 2012; López-Gómez et al., 2012).

Adhesion and invasion are thought to be key events in the pathogenesis of NTHi, and the ability to enter and survive in the intracellular space has been proposed as a means by which the organism evades both the host immune response and the inhibitory effects of antibiotics during persistent, recurrent or intractable infections (Clementi and Murphy, 2011; Hotomi et al., 2010; King, 2012; Swords et al., 2000). Bacterial attachment to the respiratory epithelia is established through adhesins, pili and proteins such as Hia and Hap factors in NTHi (St Geme, 2002). Individual strains of NTHi can produce different forms of adhesin molecules on their surfaces specific for a particular type of receptors in different cell types (King, 2012; Murphy et al., 2009). Cell receptors are a key element in bacterial attachment to the cell surface and essential for cell invasion and persistence (Plotkowski et al., 1993). β -glucan and platelet-activating factor (PAF) receptors are vital in adherence and entry of NTHi into monocytic and epithelial cells respectively (St Geme, 2002). For example, the choline moiety of bacterial lipooligosaccharide (LOS) interacts with PAF receptors for binding and internalisation into host cells (Ahren et al., 2001b; Swords et al., 2000).

NTHi entry into epithelial cells appears to be a complex process that involves bacterial adhesins, host cell receptors, signalling, endocytosis and trafficking pathways (Clementi and Murphy, 2011; López-Gómez et al., 2012). A range of outer membrane proteins and lipooligosaccharides have been identified as adhesins (Raffel et al., 2013; Swords et al., 2000), and there is some evidence to suggest that altered penicillin binding protein 3 (PBP3) may also be involved in adhesion and subsequent invasion. Two recent studies found that isolates with altered PBP3, which is associated with decreased binding affinity and susceptibility to β -lactam antibiotics, are more invasive in BEAS-2B cells than isolates with normal PBP3 (Atkins et al., 2014; Okabe et al., 2010) but the mechanism has not been clarified. The relationship between altered PBP3 and invasion is particularly concerning

because the prevalence of these strains is increasing worldwide (Kishii et al., 2011; Witherden et al., 2011).

Despite increased understanding of some mechanisms involved with invasion, the relationship between intracellular NTHi and pathogenesis is still unclear and many studies have shown enormous strain-to-strain variation in the *in vitro* invasive ability of clinical isolates (Ahren et al., 2001b; Clementi and Murphy, 2011; Goyal et al., 2015; Swords, 2012; Swords et al., 2000). More specifically, Atkins *et al.* (2014) was unable to demonstrate a significant difference in invasion between isolates from various clinical sites and those isolated from the nasopharynx of healthy carriers, and when Hotomi *et al.* (2010) studied five isolates from cases of intractable otitis media, two of these were non-invasive.

The gentamicin survival assay is the most widely used technique to determine bacterial invasion into eukaryotic cells (Edwards and Massey, 2011). Though there are a number of well-known issues with gentamicin survival assay that can influence the results potentially leading to significant errors and bias (Edwards and Massey, 2011). Factors such as time, physiological state of epithelial cells in culture and test organism, survival of eukaryotic cells, and leaking of bacteria from cells can affect the invasion assay result (Virginia and Patrik, 1994). More recently, a study investigating *Staphylococcus aureus* invasion with the SAOS-2 osteoblast-like cell line has suggested that gentamicin at a concentration of higher than 100 µg/ml can permeate the cells killing internalised bacteria *in vitro* (Mohamed et al., 2014). Cellular damage can also occur due to bacterial toxins, bacterial invasion or hindrance of normal cell function leading to the increased penetration of gentamicin into cells and elimination of internalised bacteria, thus compromising invasion assay result (Edwards and Massey, 2011).

One of the limitations in understanding the relationship between intracellular NTHi and pathogenesis, is a lack of a standardised model for studying invasion. Most studies have used a simple monoculture method where the model is restricted to epithelial and bacterial cells, whereas coculture methods, that combine epithelial, bacterial and other host cells may better reflect the *in vivo* environment (Duell et al., 2011). Clementi & Murphy (2011) acknowledges

that careful selection of both bacterial strains and host cell types for *in vitro* invasion studies is required, yet even a brief review of the literature reveals that a very large range of both respiratory and non-respiratory, and primary and immortal cell lines have been used, often without explanation or justification for the cell type used (Ahren et al., 2001b; Hotomi et al., 2010; Ketterer et al., 1999; López-Gómez et al., 2012; Okabe et al., 2010; Raffel et al., 2013; Swords et al., 2000). It is unclear whether an isolate that shows *in vitro* invasion in one cell type will be similarly invasive in another cell type, and this makes comparisons between studies very difficult.

The aim of this study is to compare the ability of a range of NTHi strains to invade a variety of respiratory cell types *in vitro* using a monoculture model and also to see if the increased invasion associated with altered PBP3 and BEAS-2B cells extends to other cell types.

3.3 Methods

3.3.1 Bacterial Strains and Culture Conditions

A collection of 16 clinical isolates of NTHi from de-identified patients was established, from the following sites and clinical conditions: ear (otitis media, n=4), eye (conjunctivitis, n=4), sputum (lower respiratory tract infection, n=5) and oropharynx (normal flora, n=3). Bacterial identity was confirmed using a PCR algorithm for *hpd*, *fucK* and *sodC* as described previously (Witherden and Tristram, 2013) and characterised as having normal or abnormal PBP3 using the PCR method of Witherden *et al.* (2011). Isolates were stored in glycerol broth in liquid nitrogen and sub-cultured at least twice on chocolate agar at 37°C in 5% CO₂ for 24 hours before use in invasion assays.

3.3.2 Epithelial Cell Lines and Cell Culture

Immortalised BEAS-2B (Sigma-Aldrich), NHBE (Lonza), A549 (Public Health England) and NCI-H292 (ATCC) epithelial cell lines were grown and maintained in LHC8 (Gibco), BEGM (Lonza), DMEM growth medium (Sigma-Aldrich) supplemented with 2Mm Glutamine and 10%

Foetal Bovine Serum and RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS), respectively at 37°C in 5% CO₂.

3.3.3 Invasion Assay

The invasive ability of the different NTHi strains was determined for each epithelial cell line using the gentamicin survival assay as described previously by Atkins *et al.*, (2014). Briefly, bacterial isolates grown overnight on chocolate agar were used to prepare bacterial suspensions in corresponding growth medium at a concentration of approximately 10⁶ bacteria ml⁻¹. BEAS-2B, NHBE, A549 and NCI-H292 epithelial cells were seeded in a 24-well collagen coated cell culture plates (ThermoFisher Scientific) at a density recommended by supplier per well and were incubated for approximately 48 hours until greater than 90% confluence was achieved. Monolayers were washed twice with their respective pre-warmed culture medium before exposure to NTHi strains. Monolayers were inoculated with the NTHi strains in triplicate at 10⁶ bacteria per well and incubated for 4 hours after which the monolayers were washed three times with Hank's Buffered Salt Solution (HBSS). Pre-warmed cell culture medium containing 200 µg gentamicin ml⁻¹ was added to all 24 wells and incubated for 2 hours to kill extracellular bacteria. Monolayers were then washed three times with HBSS after which intracellular bacteria were released by lysing the cells with 1% Saponin in HBSS for 15 min, and collected by scraping and vigorous vortexing for 1 min. The lysates were serially diluted and spread on chocolate agar in duplicate. The invasion rate was calculated by counting the c.f.u. and calculating a % invasion in relation to the original inoculum. The assay for each strain was conducted in triplicate on three different days.

3.3.4 Statistical Analysis

All data analysis was conducted using GraphPad Prism software (version 6.0d). A Shapiro-Wilks test was performed to assess the normality or otherwise of the distribution of invasion rates across the isolates. Analysis of the differential invasion rate of all strains in the four

different cell types (BEAS-2B, NHBE, A549 and NCI-H292) was conducted using a Kruskal-Wallis test and the analysis of the invasion rate of each individual strain over the four cell types was conducted using a two-way ANOVA. A Mann-Whitney test was performed to analyse the difference in invasion rate between strains with normal and altered PBP3 for each cell type. P values <0.05 were considered as statistically significant.

3.4 Results

The invasion rates were not normally distributed across strains, so non-parametric tests were selected for subsequent analysis. The invasion rates for all 16 isolates with all four epithelial cell types are given in Table 3.1 and shown in Figure 3.1. The range and median invasion rates for all isolates across all cell types was < 0.01 to 43.45% and 1.10%, and individually for BEAS-2B cells was 0.03 to 43.45% and 3.17%, for NHBE cells was 0.10 to 13.99% and 2.31%, for A549 cells was < 0.01 to 8.98% and 0.11%, and for NCI-H292 cells was 0.23 to 12.72% and 1.52%. The median invasion with BEAS-2B cells was significantly higher compared to A549 cells ($P = 0.015$), however, there was no significant difference when comparing to NHBE or NCI-H292 cells, and the median invasion with A549 cells was significantly lower than NCI-H292 ($P = 0.015$) cells but did not exhibit any statistical difference with NHBE cell types (Figure 3.2). There were marked differences in invasion rates for some isolates depending on the cell type used, where for example, isolate L341 gave 43.45% invasion in BEAS-2B cells but only 3.63% in A549 cells, L267 ranged from 22.12% (BEAS-2B) to 2.87% (A549) and Ci51 from 18.01 % (BEAS-2B) to 1.68% (A549). Alternatively, some isolates showed more consistent invasion rates, such as Ci34 with $< 0.01\%$ (A549) to 0.23% (NCI-H292), Ci24 with 0.04% (A549 and BEAS-2B) to 0.50% (H292) and Ci35 with $< 0.01\%$ (A549) to 0.54% (NCI-H292). For individual cell types BEAS-2B, NHBE, A549 and NCI-H292, the median invasion rates for isolates with normal PBP3 were 0.07, 1.92, 0.02, and 1.10 respectively, compared to 9.90, 2.70, 1.68 and 3.66 respectively for those with altered PBP3. The median invasion rates were higher for isolates with altered PBP3 compared to normal PBP3 in all cell types, and this was

statistically significant for BEAS-2B cells ($P=0.031$) and A549 cells ($P=0.037$) but not for NHBE or NCI-H292 (Figure 3.3).

Table 3.1 Comparative mean invasion rate (%) of NTHi isolates by cell type (submitted as supplementary data in the associated publication).

NTHi Strain identifier	% Mean Invasion Rate				Altered PBP3
	BEAS-2B	NHBE	A549	NCI-H292	
Ci5	1.71	1.92	0.02	3.37	No
Ci8	4.63	5.47	0.17	1.54	No
Ci24	0.04	0.05	0.04	0.50	No
Ci35	0.03	0.10	< 0.01	0.54	No
Ci34	0.07	0.02	< 0.01	0.23	No
NF3	10.94	6.77	0.29	2.83	No
J76	0.03	2.78	< 0.01	1.10	No
Ci49	0.85	0.54	< 0.01	0.72	Yes
Ci51	18.01	13.99	1.68	3.86	Yes
Ci11	11.49	0.83	8.98	1.50	Yes
Ci17	0.52	0.40	0.05	3.66	Yes
L60	0.21	1.08	0.03	1.06	Yes
L236	9.90	2.70	0.27	0.83	Yes
L267	22.12	5.91	2.87	12.72	Yes
L227	9.08	3.70	2.45	10.97	Yes
L341	43.45	11.98	3.63	7.75	Yes

BEAS-2B: Immortalised bronchial epithelial cells. NHBE: Primary bronchial epithelial cells. A549: Lung carcinoma cells. NCI-H292: Muco-epidermoid lung carcinoma cell.

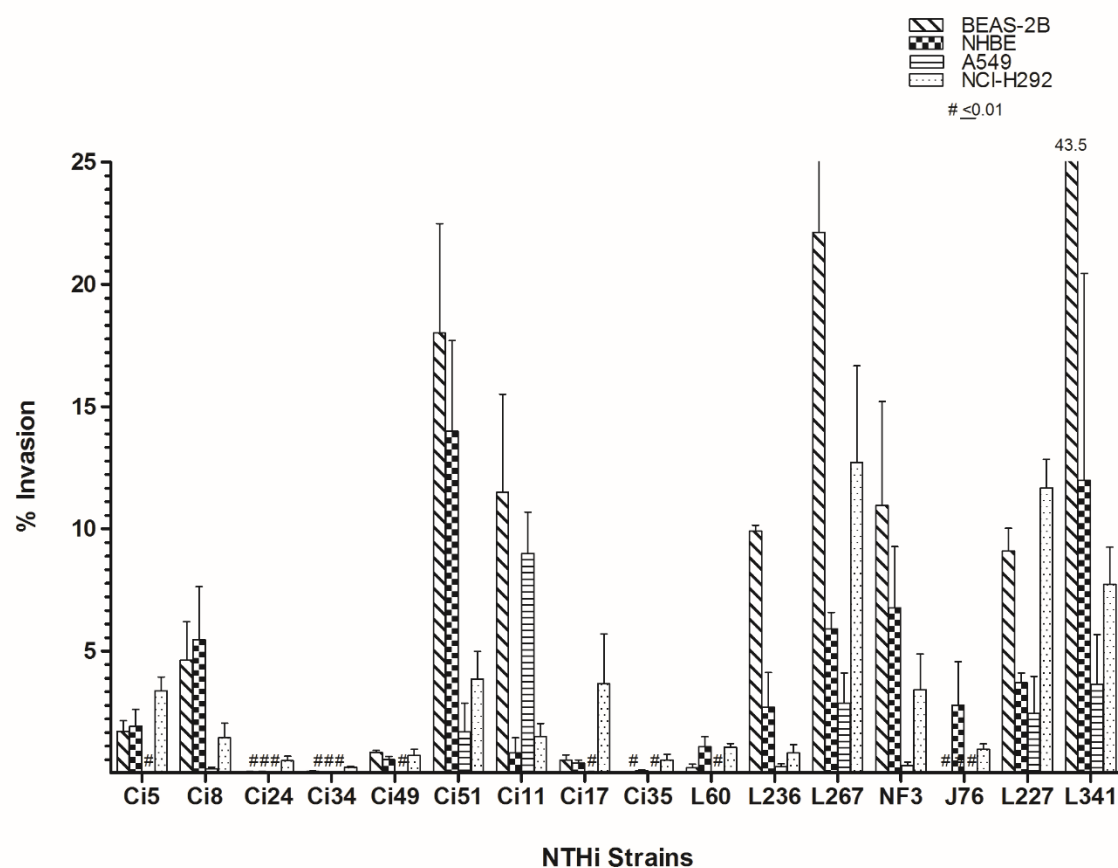


Figure. 3.1 Comparative mean invasion rates of individual NTHi isolates in BEAS-2B, NHBE, A549 and NCI-H292 cells (*submitted as supplementary data in the associated publication*). Bars represent the mean of 3 individual assays \pm SEM. # Invasion < 0.01%.

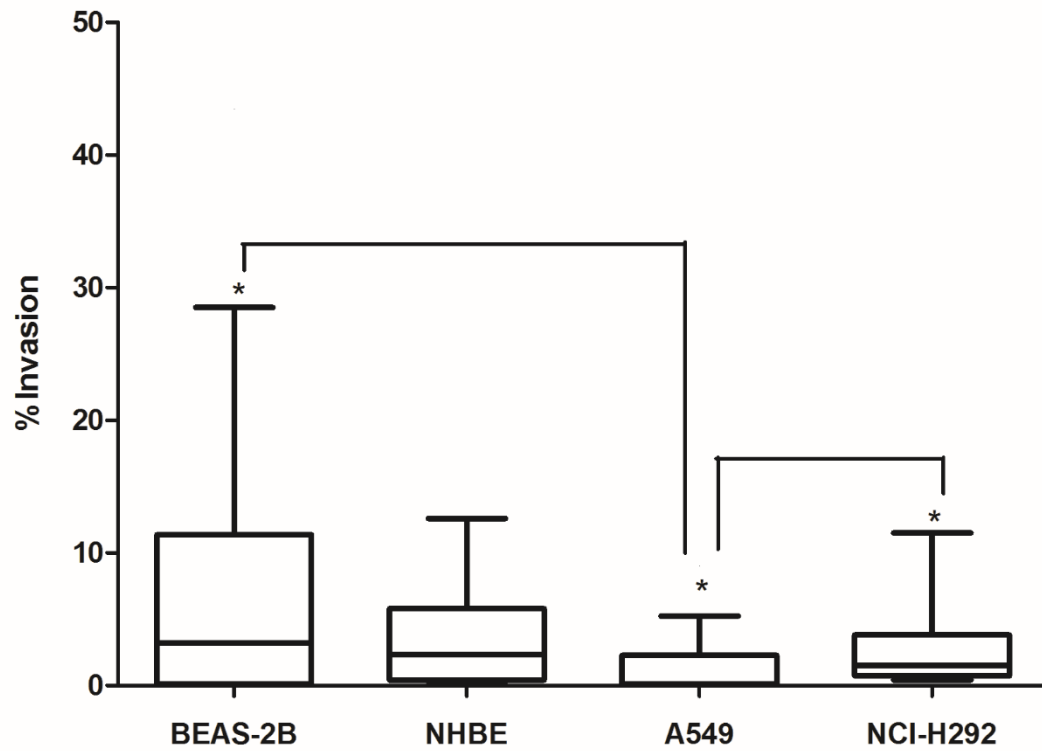


Figure 3.2 Box-plot of invasion and epithelial cell type

Box lines represent median, 25th and 75th percentile and entire data range.

* represents significant difference ($p < 0.05$)

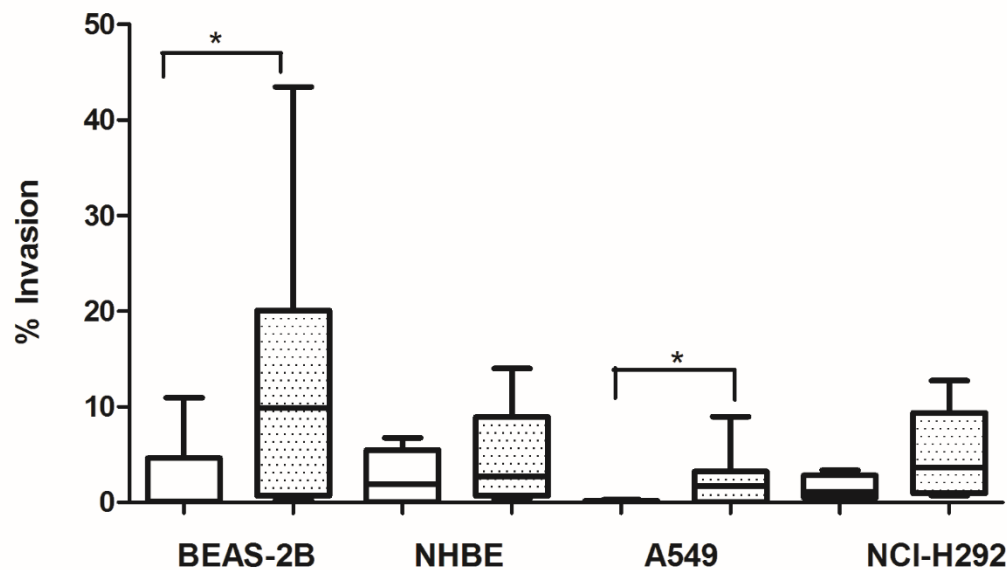


Figure 3.3 Comparative invasion between normal and abnormal PBP3
 Empty box represents normal PBP3, dotted box represents abnormal PBP3.
 Box lines represent median, 25th and 75th percentile and entire data range.
 * represents significant difference ($p < 0.05$)

3.5 Discussion

The ability of NTHi to invade and survive intracellularly has been suggested as one of the means to avoid host immune response and the effects of antibiotic treatment providing a reservoir for re-infections (Clementi and Murphy, 2011; King, 2012; Swords et al., 2000). The gentamicin survival assay was selected because it has been widely used for studying *in vitro* invasion with NTHi, including the studies of Okabe *et al.* (2010) and Atkins *et al.* (2014) to which we wish to compare, and because it is able to generate reproducible quantitative data suitable for our purpose (Edwards and Massey, 2011). We chose a range of respiratory epithelial cells because these have been more frequently used to study the association between *in vitro* invasion and pathogenicity than non-respiratory cells.

Not only did our study show significant differences in invasion for the isolates collectively across different cells types, but in some instances this occurred for individual isolates between different cell lines from the same anatomical niche. For example, the invasion of isolate Ci11

was very different between both bronchial cells lines and also between both lung cells lines and underlines the difficulty in comparing results between studies. Our results also show that it is meaningless to categorise isolates as “invasive” or “non-invasive” using *in vitro* models because there is no standard invasion rate accepted for defining an isolate as “invasive”, and even if an arbitrary definition is applied, it cannot account for the effect of respiratory cell type. In the study by Okabe *et al.* (2010), an arbitrary “cut-off” of > 1% invasion was used to designate an isolate as “invasive”, yet in our study, isolates Ci5, L60 and J76 would be classified as “invasive” using some epithelial cells and “non-invasive” using others.

A possible limitation in our study is that we did not investigate epithelial cell viability after the 4hr incubation with NTHi. If there was any significant epithelial cell death or damage due to bacterial induced cytotoxicity, this may lead to falsely low invasion rates: either because of release of intracellular bacteria and subsequent killing by gentamicin, or because of increased permeability of the damaged cells to gentamicin and killing of intracellular bacteria. However, a recent study has shown that NTHi 86-028NP had minimal impact on the viability of A549 and D562 cells, even after 24 hours, although it is not known if this would extend to other strains of NTHi or to other epithelial cell types (Pickering *et al.*, 2016).

We did not attempt to identify the mechanisms that may be responsible for the differences in invasion between isolates and/or cell types but our results show that this may be useful in understanding the mechanisms of invasion. For example, are some bacterial cell associated adhesins more important for the invasion of specific cell lines, and are specific cell associated receptors more important for invasion by specific bacterial isolates?

Our finding of increased median invasion of BEAS-2B cells in isolates with altered PBP3 is consistent with the original finding of Okabe *et al.* (2010) and the subsequent finding of Atkins *et al.* (2014), who extended the knowledge in this area by also comparing the invasion of otherwise isogenic strains and showing that the invasion rates of isolates with normal PBP3 could not be increased by artificially introducing altered PBP3. This suggested that the increased invasion associated with isolates with altered PBP3 was not directly due to the altered PBP3 but this has not been further investigated. The finding in this current study that

the increased *in vitro* invasion of BEAS-2B cells for isolates with altered PBP3 extends to other respiratory cell types is significant because it raises the importance of this issue beyond a curious finding, to one that offers useful avenues for additional investigation. Isolates with altered PBP3 are increasing worldwide, with recent studies showing a prevalence of 20% in Australia (Witherden et al., 2011), 40% in Spain (Puig et al., 2013) and over 60% in Japan (Kakuta et al., 2016), where such strains are particularly common, and this may provide an avenue to indirectly assess whether invasion as demonstrated by *in vitro* studies, is associated with pathogenicity. Acute otitis media is commonly caused by NTHi, and the frequent recurrence or requirement for prolonged therapy in Japan has previously been attributed to the prevalence of isolates with altered PBP3 and their associated decreased susceptibility to β -lactam antibiotics and compromised treatment (Hoshino et al., 2013; Kakuta et al., 2016; Yamanaka et al., 2008). Our findings suggest that the recurrent or chronic nature of the otitis media in that context may at least in part be due to the increased probability that strains with altered PBP3 can sequester in the intracellular space. However, a different view is presented by a study by Hagiwara *et al.* (2012), where NTHi isolates were collected from the sputa of adults with a variety of respiratory diseases. Of the 144 isolates studied in detail, 51% had altered PBP3, yet the pathogenicity of those isolates, as measured by clinical findings and a range of laboratory determined inflammatory markers did not differ from those with normal PBP3 (Hagiwara et al., 2012).

In conclusion, our study showed that the ability of NTHi to invade respiratory epithelial cells *in vitro* is both strain dependant and influenced significantly by the respiratory cell line used, and that the association between altered PBP3 and increased invasion is conserved across multiple cell lines.

Chapter 4.0 – Manuscript 2

Inability of *H. haemolyticus* to invade epithelial cells *in vitro*

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4.1 Abstract

Nontypeable *Haemophilus influenzae* (NTHi) is a commensal of the upper respiratory tract and an important opportunistic pathogen, causing both acute and chronic respiratory infections. Many isolates of NTHi have been shown to be able to invade respiratory epithelial cells *in vitro*, and it has been suggested that this may be a virulence attribute, particularly in persistent infections where the intracellular environment would protect the bacterial cells from both the host immune response and antibiotic exposure. *H. haemolyticus* is very closely related to NTHi and shares the same upper respiratory tract habitat but is not an opportunistic respiratory pathogen. Little information is available on the ability of *H. haemolyticus* to invade epithelial cells *in vitro*. The aim of this study was to investigate the difference in ability of NTHi and *H. haemolyticus* to invade respiratory epithelial cells *in vitro*. A collection of *H. haemolyticus* (n=20) all from the throats of healthy volunteers was established, and molecular methods used for identification. A gentamicin survival assay was used to determine the invasive ability of isolates *in vitro*, with results expressed as a percentage of bacterial cells from the challenge inoculum that were recovered from the BEAS-2B intracellular space after 4 hours co-incubation. The results demonstrated the inability to invade with invasion rate of <0.01% for all 20 isolates tested with BEAS-2B cells and five selected isolates with NHBE, and A549 cells. Invasion rates ranged from 0.03 to 0.67 with NCI-H292 cells, but are still under the criteria of “non-invasive”. This confirms that inability of *H. haemolyticus* isolates to invade *in vitro* model is consistent with their non-pathogenic nature.

4. 2 Introduction

Nontypeable *Haemophilus influenzae* is an opportunistic human pathogen responsible for diseases such as exacerbation of chronic bronchitis, community acquired pneumonia, otitis media and occasionally conjunctivitis. The factors associated with pathogenicity of NTHi are complicated, and include antibiotic resistance, biofilm formation, mucociliary interactions, attachment to respiratory mucosa, evasion of mucosal immunity and invasion. Invasion allows

the bacteria to enter into host cells and is initiated via adherence of bacteria to the cell surface (Baron S, 1996; King, 2012). Some NTHi strains possess increased ability to invade respiratory epithelial cells; this may be a way to avoid the host's normal immune response and finally use it as a persistent reservoir for infection (King, 2012).

Haemophilus haemolyticus is very closely related to NTHi and shares the same upper respiratory tract habitat but is not as an opportunistic respiratory pathogen. NTHi and *H. haemolyticus* can easily be distinguished from other *Haemophilus* species due to their requirements for both X and V blood derivatives for their *in vitro* growth. However, discriminating these two on phenotypic basis is a tedious task, as they possess very similar colony morphology and biochemical activity. Traditionally, haemolytic activity on horse blood agar from *H. haemolyticus* could be used to differentiate these two species. However, with the identification of non-haemolytic strains of *H. haemolyticus*, the reliability of this test is now limited (Murphy et al., 2007; Sandstedt et al., 2008). Retrospective analysis by molecular detection methods has confirmed in a number of instances where *H. haemolyticus* was erroneously identified as NTHi (Murphy et al., 2007). Re-identification of 490 NTHi isolates by molecular methods revealed that 39.5% (102/156) of NTHi isolates from COPD patients and 27.3% (12/32) NTHi isolates recovered from the nasopharynx of children were actually non-haemolytic *H. haemolyticus* (Murphy et al., 2007). Other studies also reported the similar misidentification; 20.8% of presumptive NTHi from the throats of children and 16-21% of presumptive NTHi from lung specimens were actually *H. haemolyticus* (Mukundan et al., 2007; Xie et al., 2006). Although *H. haemolyticus* has been reported as commensal as it has occasionally been recovered from sterile sites (Hinz et al., 2015; Mukundan et al., 2007; Pickering et al., 2016), which raises the question of whether it is truly non-pathogenic?

Both NTHi and *H. haemolyticus* can acquire resistance to β -lactam antibiotics via mutations and associated amino acid substitutions in penicillin binding protein 3 (PBP3) and the prevalence of such resistant strains is increasing worldwide (Tristram et al., 2007; Witherden et al., 2014). There are a number of studies confirming that *H. haemolyticus* isolates possess mutated *ftsI* genes, encoding for altered PBP3. Takahata et al. (2007) demonstrated the

horizontal transfer of partial fragments of mutated *ftsI* gene from *H. haemolyticus* strains into NTHi and suggested this as one of the essential factors for such a rapid increase of these altered PBP3 strains worldwide. Witherden *et al.* (2014) observed the exchange of partial fragments of mutated *ftsI* gene between NTHi and *H. haemolyticus* where the mosaic regions of *ftsI* sequences actually originated from *H. haemolyticus* in 36% of NTHi strains and from NTHi in 32% of *H. haemolyticus* strains.

Recently, two studies have demonstrated that NTHi strains with altered PBP3, which is responsible for reduced susceptibility and binding affinity to β -lactam antibiotics, possess more invasive ability compared to strains with normal PBP3 but the mechanism has not been investigated (Atkins *et al.*, 2014; Okabe *et al.*, 2010). NTHi invasion and persistence in respiratory epithelial cells have been shown in many studies and suggested as one of the contributors to the pathogenicity of NTHi *in vivo* (King, 2012; Clementi and Murphy, 2011; Atkins *et al.*, 2014). The correlation between local invasion and pathogenesis is still a matter of debate, but NTHi infections often recur and persist even after successful antibiotic therapy and clinically negative culture outcomes (King, 2012). While the association between altered PBP3 and increased invasion has been demonstrated in NTHi, very little information is available on the ability of *H. haemolyticus* to invade epithelial cells *in vitro* (Pickering *et al.*, 2016) and has not been investigated if there is any difference between invasive ability of altered and normal PBP3 strains of *H. haemolyticus*.

If *in vitro* invasion is an indicator of ability for *in vivo* invasion and is important in the pathogenesis of NTHi infection, then we hypothesise that isolates of *H. haemolyticus* will be comparatively non-invasive.

4.3 Methods

4.3.1 Bacterial Strains and Culture Conditions

A collection of 20 *H. haemolyticus* isolates, recovered from normal throat flora of healthy individuals, was established. Isolates were identified as *H. haemolyticus* based on positive

PCR for *sodC* and negative PCR for *hpd* and *fucK* using the methods and algorithm as described previously (Witherden and Tristram, 2013). The nature of penicillin binding protein 3 (PBP3) in the isolates was investigated using PCR to identify single nucleotide *ftsI* polymorphisms responsible for altered PBP3 with substitution of asparagine to lysine at position 526 (N526K) as described previously (Witherden et al., 2013). In our 20 isolates, 12 isolates were detected to have altered PBP3 and 8 isolates were with normal PBP3. Isolates were stored in glycerol broth in liquid nitrogen and sub-cultured at least twice on chocolate agar at 37°C in 5% CO₂ for 24 hours before use in invasion assays. A highly invasive NTHi isolate L341 has been used as a positive control for this study (see table 3.1).

4.3.2 Epithelial Cell Lines and Cell Culture

Immortalised BEAS-2B (Sigma-Aldrich), NHBE (Lonza), A549 (Public Health England) and NCI-H292 (ATCC) epithelial cells were grown and maintained in LHC8 (Gibco), BEGM (Lonza), DMEM growth medium (Sigma-Aldrich) supplemented with 2Mm Glutamine and 10% Foetal Bovine Serum and RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% foetal bovine serum (FBS), respectively at 37°C in 5% CO₂.

4.3.3 *H. haemolyticus* Invasion with Multiple Cell Types

The intracellular survival of *H. haemolyticus* isolates was determined using gentamicin survival assay as described previously by Atkins *et al.*, (2014) and used above mentioned invasive NTHi isolate as positive control. The detailed procedure of the gentamicin survival assay is discussed in chapter 2 of this thesis. This assay was performed across each cell type used in this study.

4.4 Results

Results are expressed as a percentage of bacterial cells from the challenge inoculum that were recovered from the BEAS-2B intracellular space after 4 hours co-incubation. All 20

isolates had invasion rates of < 0.01% (table 4.1). To determine if this “non-invasiveness” extended to other cell lines, we similarly tested five selected isolates (two normal PBP3 and three altered PBP3) for invasion using NHBE, A549 and NCI-H292 cell lines. All isolates had invasion rates of < 0.01% in NHBE and A549 cells (table 4.2), but invasion rates ranged from 0.03 to 0.67 for NCI-H292, although this is still less than the > 1% that Okabe *et al.* (2010) uses as criteria for invasion. In summary, we have shown that our population of *H. haemolyticus* isolates are non-invasive.

Table 4.1 Mean invasion rate (%) of *H. haemolyticus* isolates with BEAS-2B cell type.

Hh Strain Identifier	% Mean Invasion with BEAS-2B Cells	Altered PBP3
L3	<0.01	No
L4	<0.01	Yes
L8	<0.01	Yes
L12	<0.01	No
L23	<0.01	Yes
L42	<0.01	No
L47	<0.01	No
L48	<0.01	Yes
L51	<0.01	Yes
L54	<0.01	Yes
L55	<0.01	No
L56	<0.01	Yes
L61	<0.01	No
L66	<0.01	Yes
L73	<0.01	No
L95	<0.01	No
L187	<0.01	Yes
L190	<0.01	Yes
L191	<0.01	Yes
L320	<0.01	Yes

BEAS-2B: Immortalised bronchial epithelial cells

Table 4. 2 Comparative mean invasion rate (%) of five selected *H. haemolyticus* isolates with NHBE, A549 and NCI-H292 cell types.

Hh Strain Identifier	% Mean Invasion			Altered PBP3
	NHBE	A549	NCI-H292	
L47	<0.01	<0.01	0.08	No
L48	<0.01	<0.01	0.03	Yes
L51	<0.01	<0.01	0.33	Yes
L54	<0.01	<0.01	0.67	Yes
L55	<0.01	<0.01	0.04	No

NHBE: Primary bronchial epithelial cells. A549: Lung carcinoma cells. NCI-H292: Muco-epidermoid lung carcinoma cell

4.5 Discussion

Invasion of respiratory epithelial cells is thought to be an essential factor in the pathogenesis of NTHi. However, the clinical significance of invasion in pathogenicity of NTHi is not yet clear. The results from our study provide some additional insight into what is already known about *in vitro* invasion in NTHi. Although previous work by Atkins *et al.* (2014) showed that *in vitro* invasion was not an essential attribute for pathogenesis, as many isolates in that study being clinical isolates were non-invasive, the observation that none of 20 *H. haemolyticus* isolates tested was invasive supports the notion that *in vitro* invasion may be associated with some aspect of *in vivo* pathogenicity. Similarly, Atkins *et al.* (2014) showed that although altered PBP3 was associated with increased *in vitro* invasion, altered PBP3 alone was unable to enhance *in vitro* invasion in non-invasive NTHi isolates. Our demonstration that altered PBP3 did not have any effect on invasiveness in *H. haemolyticus* supports the conclusion of Atkins *et al.* (2014) that PBP3 is not directly associated with invasion.

We did not attempt to identify the mechanisms that may be responsible for such differences in invasion rate between NTHi and *H. haemolyticus* isolates but our results show that this may be useful in understanding the relationship between invasion and pathogenicity. For example, what specific attributes do pathogenic NTHi isolates possess that are lacking in non-invasive *H. haemolyticus* isolates?

One of the possible limitations of our study is that we did not test epithelial cell viability after the 4hr incubation with *H. haemolyticus*. If there was any significant epithelial cell death or damage due to bacterial induced cytotoxicity, this may lead to falsely low invasion rates due to release of intracellular bacteria and subsequent killing by gentamicin, or because of increased permeability of the damaged cells to gentamicin and killing of intracellular bacteria. A recent study has shown that *H. haemolyticus* can cause significant reduction in viability of A549 and D562 cells over the period of 24 hours, however there was only a 15% reduction in viability of A549 cells with no impact on D562 cells viability after 3 hours, which is the usual incubation time frame for gentamicin survival assay, and it is not known if this would extend to another epithelial cell types (Pickering et al., 2016). Other potential limitation of our study is that we did not test the viability of *H. haemolyticus* in relative cell culture media for different cell types used in our study as it does not grow well in liquid media and compromised viability can lead to false invasion assay results, however the study by Pickering *et. al.*, (2010) showed relatively stable viability in A549 and D562 cell culture media for the first 3 hours of incubation. The molecular mechanism of invasion in NTHi has not been fully elucidated (Clementi and Murphy, 2011; Ikeda et al., 2015). Our demonstration of non-invasiveness in *H. haemolyticus* may provide a novel avenue to study the genetic determinants of invasion. As *H. haemolyticus* and NTHi are phylogenetically very closely related (Price et al., 2015; Witherden et al., 2013), a genome wide comparison between *H. haemolyticus* and both invasive and non-invasive NTHi may reveal key genomic regions associated with invasion. A similar approach has been used previously to identify the genomic regions associated with NTHi that cause otitis media by comparing isolates from cases of otitis media with isolates from the throat of non-diseased individuals (Xie et al., 2006).

In conclusion, we have shown that our collection of *H. haemolyticus* isolates are non-invasive using an *in vitro* model and suggest that this is consistent with their inability to cause opportunistic respiratory tract infections.

Chapter 5.0 – General Discussion

5.1 Summary of Thesis and Major Findings

Adhesion and invasion of NTHi in respiratory epithelial cells are thought to be supporting factors in its pathogenicity. It has been suggested that the ability of NTHi to survive intracellularly allows NTHi to avoid the host immune response and the effects of antibiotic treatment, and to use the intracellular space as a reservoir for persistent, recurrent and intractable infections. The mechanism of invasion is a highly complex process which involves factors such as bacterial adhesins, host cell receptors, signalling, endocytosis and trafficking pathways. These factors can vary from one cell type to another and may have caused the variability in invasion rate results between studies, as a very large range of both respiratory and non-respiratory, and primary and immortal cell lines have been used for invasion assays without any explanation or justification. It is unclear whether an isolate that shows *in vitro* invasion in one cell type will be similarly invasive in another cell type, and this makes comparisons between studies very difficult.

H. haemolyticus is a respiratory commensal that is closely related to NTHi and shares the same respiratory niche, but is not considered to be an opportunistic respiratory pathogen. Both NTHi and *H. haemolyticus* can acquire resistance to β -lactam antibiotics via mutations and associated amino acid substitutions in PBP3. Recently, it has been suggested that altered PBP3 can enhance adhesion and subsequent invasion in NTHi, but it is not clear if this is the case in *H. haemolyticus*. The research projects comprising this thesis involve the investigation of the invasion rate of NTHi and *H. haemolyticus* across a range of cell types used in this study.

The gentamicin survival assay was selected because it has been frequently used to investigate *in vitro* invasion with NTHi, including the recent studies by Okabe *et al.* (2010) and Atkins *et al.* (2014), which prompted the current investigation, and because it is able to produce quantitative data suitable for the study purpose. We investigated the invasion rate

with a range of respiratory epithelial cell lines that have been widely used to explore the association between *in vitro* invasion and pathogenicity in NTHi.

This thesis for the first time demonstrates that invasion not only varies for the isolates collectively across different cell types but in some cases, it also varies for individual isolates between different cell lines from the same anatomical niche. For example, isolate Ci11 exhibited very different invasion rates from one cell type to another. This variability in invasion rates from one cell type to another emphasises the difficulty in interpreting the results between studies. This study also showed that the categorisation of the isolates as “invasive” or “non-invasive” using *in vitro* models is irrelevant, because there is no such standard invasion rate distinction established to classify an isolate as “invasive”. Even if an arbitrary “cut-off” of $\geq 1\%$ provided by Okabe *et al.* (2010) is applied, it cannot be applicable to all the cell types due to lack in consistency of invasion rates from one cell type to another, where some isolates such as Ci5, L60 and J76 would be classified as “invasive” using some epithelial cells and “non-invasive” using others.

The other important finding from the work presented in this thesis is that NTHi isolates with altered PBP3 possess increased invasive ability with BEAS-2B cells compared to isolates with normal PBP3 which is consistent with the findings of Okabe *et al.* (2010) and the successive study by Atkins *et al.* (2014). This increased invasiveness due to altered PBP3 also extends to other cell types used in this study. This is an important finding because it adds more insight to this topic and offers useful avenues for further investigation. Isolates with altered PBP3 are increasing worldwide and where diseases due to such strains are particularly common, this may provide an avenue to indirectly assess whether invasion, as demonstrated by *in vitro* studies, is associated with pathogenicity.

This thesis also demonstrated the inability of *H. haemolyticus* to invade across all four cell types used (chapter 4) and added some further insight into what is already known about *in vitro* invasion in NTHi. The previous work by Atkins *et al.* (2014) provided some evidence that *in vitro* invasion was not an absolute pre-requisite for pathogenicity, as a number of NTHi isolates from clinical disease were not invasive, and there was no statistically significant

difference in *in vitro* invasion between isolates from various clinical diseases and those isolated as normal flora from non-diseased individuals. However, the findings in this study, that none of the isolates of the “non-pathogenic” *H. haemolyticus* show *in vitro* invasion tends to support some association between *in vitro* invasion and *in vivo* pathogenic ability. The finding of no difference in invasive ability between NTHi isolates with normal PBP3 or altered PBP3 supports the notion that altered PBP3 alone is not directly associated with increased *in vitro* invasion. This suggests the presence of another exclusive factor in such strains which is responsible for increased invasiveness.

5.2 Limitations of Thesis and Future Directions

A possible limitation of this study is that the effect of epithelial cell viability during the gentamicin survival assay was not investigated. If there was any significant epithelial cell death or damage due to bacterial induced cytotoxicity, this may lead to falsely low invasion rates (Edwards and Massey, 2011). However, a recent study showed that *H. haemolyticus* had minimal impact on the viability of A549 at 3 hours’ time point, although it is not known if this would extend to other epithelial cell types. Future studies need to investigate the impact of NTHi and *H. haemolyticus* on viability of other cell types as a result for better validation of invasion results.

We also did not attempt to identify the mechanisms that may be associated with the differences in invasion between isolates and/or cell types. This may be useful avenue in understanding the mechanisms of invasion. For example, are some bacterial cell associated adhesins more important for the invasion of specific cell lines, and are specific cell associated receptors more important for invasion by specific bacterial isolates?

The molecular mechanism associated with invasion in NTHi is still not completely understood. Our demonstration of the inability of *H. haemolyticus* to invade respiratory cell types may provide a novel attribute to investigate the genetic elements involved in invasion. Phylogenetically, *H. haemolyticus* and NTHi are very closely related to each other and a

genome wide comparison between *H. haemolyticus* and both invasive and non-invasive NTHi may reveal key genomic regions associated with invasion.

5.3 Conclusions

In conclusion, the research findings in this thesis increase our current understanding of *in vitro* invasion by providing new information that may help to reveal the relationship between pathogenicity and invasion of NTHi.

Our key findings were:

- Choice of cell type in invasion assays is a confounding factor in *in vitro* invasion studies, as the ability of NTHi to invade respiratory epithelial cells *in vitro* is both strain dependant and influenced significantly by the respiratory cell types used. This is important because a range of epithelial cells are in use for *in vitro* invasion studies and such variability in invasion rate with different cell types emphasises the difficulty in comparing the results between different studies and the need for a standardised model for invasion assays.
- The association between altered PBP3 and increased invasion has been strengthened by this study and extended to a range of respiratory epithelial cell types used. This is an important finding because the prevalence of β -lactam-resistant strains due to altered PBP3 is increasing worldwide and in situations where patients are infected by such strains, this finding may be an important consideration in guiding therapy in these patients.
- This study also demonstrated the inability of *H. haemolyticus* isolates to invade respiratory epithelial cell types *in vitro*, which appears to be consistent with their inability to cause opportunistic respiratory tract infections. This is a novel finding and suggests that there is a link between *in vitro* invasion and *in vivo* pathogenicity. This finding may provide an avenue to uncover the relationship between invasion and pathogenicity in NTHi, which is still a matter of debate.

Chapter 6.0 – References

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